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Effect of Injection of Rat Bone Marrow on Recticular Tissues of Mice Exposed to X Radiation in the Midlethal Dose Range [1,2]

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Mice exposed to a supralethal dose of X radiation (*ca.* 900 r; total body) recovered from the radiation injury when given an intravenous injection of rat bone marrow after irradiation (*1–3*). On the other hand, all (C3H × 101)F_1 mice exposed to a midlethal dose (710 r; total body) and similarly injected died in 16 days (*4*). Thirty percent of the mice exposed to 710 r but receiving no marrow injection died in 30 days. None of the mice injected with isologous bone marrow after 710 r died.

In this report, we shall discuss the histologic changes in the reticular tissues of mice receiving 710 r of X radiation and rat bone marrow compared with those receiving 710 r alone or 710 r and isologous bone marrow.

Materials and Methods

Animals.—(C3H × 101)F_1 male and female mice, 12 weeks old, were divided into 4 groups. In the first group, 23 animals received 710 r of total-body X radiation and, within 4 hours, an intravenous injection of bone-marrow cell suspension taken from the humerus and the femur of a Sprague-Dawley rat weighing about 200 gm. Eleven of the mice were killed and autopsied, 1 a day from day 1 through day 11. Twelve animals that died 8 to 13 days after irradiation were also autopsied. The second group of 20 mice received 710 r and an intravenous injection of isologous mouse bone marrow within 4 hours after irradiation. Each mouse received the contents of the shaft of 1 femur from a donor mouse. All these animals were killed and autopsied 1 to 30 days after irradiation. Twenty mice in the third group were exposed to 710 r only and were killed and autopsied at the same intervals as group 2. A fourth group of 20 mice were not irradiated but received the same amount of rat bone-marrow cell suspension intravenously as group 1. They were killed and autopsied on the same schedule as mice of groups 2 and 3. In each group, the sexes were alternated at sacrifice.

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[2] Work done under USAEC Contract No. W-7405-eng-26.
[3] Public Health Service Research Fellow of the National Cancer Institute, National Institutes of Health, U. S. Department of Health, Education, and Welfare.

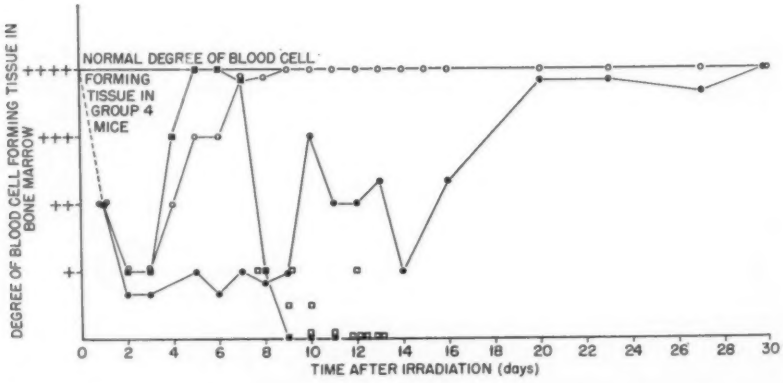
At autopsy, gross inspection of the organ systems, except the central nervous system, was made and the reticular tissues were fixed in Zenker-formol solution. Sections were prepared and stained with hematoxylin and eosin.

Irradiation conditions.—A General Electric Maxitron machine was the X-ray source. It was operated at 250 kvp, 30 ma., 3 mm. of Al filter, and at 80 cm. gave a dose rate of about 100 r per minute. The mice were exposed in a circular, perforated lucite container attached to a revolving turntable.

Results

Bone Marrow

A section from the femur and the sternum of each autopsied mouse was examined. The amount of blood-cell-forming tissue on the histologic sections was estimated. A normal, fully packed bone marrow was considered 4+. Lesser amounts of bone marrow were estimated as 3+, 2+, 1+, and total absence of blood-cell formation.[4] Text-figure 1 shows the results on the 4 groups of mice. The quick recovery of cellularity in bone marrow of irradiated animals treated with isologous bone marrow, in comparison with the slow recovery in untreated, irradiated mice, follows the expected pattern. The bone marrow of the irradiated mice treated with rat bone marrow showed an early recovery of all blood-forming elements; however, the abrupt total destruction of the marrow 6 to 9 days after irradiation precedes the characteristic time of death in these mice. Total or near-total destruction of bone marrow was found in mice that died 8 to 13 days after irradiation and treatment with rat mar-



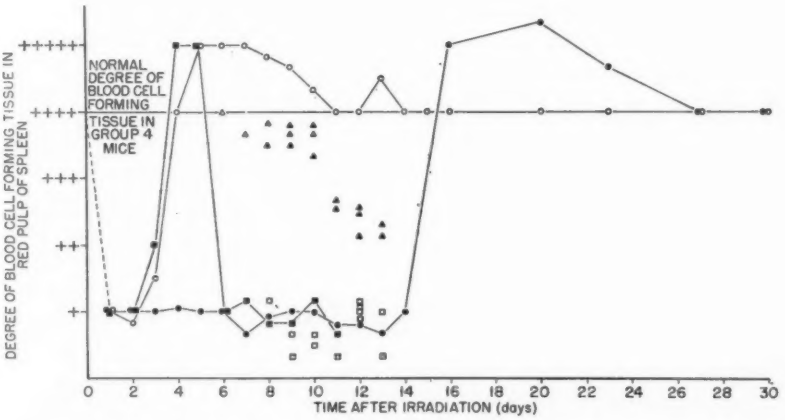
TEXT-FIGURE 1.—Effect of irradiation and bone-marrow injection on amount of blood-forming tissue in bone marrow of individual serially sacrificed (C3H × 101)F_1 mice. ■ 710 r + rat bone marrow; ○ 710 r + isologous bone marrow; ● 710 r only; □ animal died after 710 r + rat bone marrow.

[4] In the text-figures for the different tissues, intermediate degrees of cellularity between 0, 1+, 2+, etc., are also used. Small differences recorded in scoring the histologic sections are not necessarily significant.

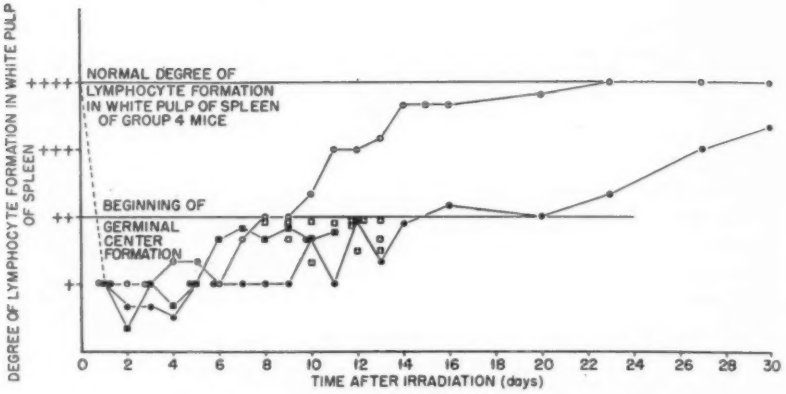
row. The histology of the bone marrow at selected intervals in these experiments is shown in figures 1 to 4.

Spleen

Sections of spleen in the 4 groups of mice were studied for blood-cell-forming tissue in the red pulp and degree of lymphocyte formation in white pulp (text-figs. 2, 3). Normal histologic character was arbitrarily recorded as 4+, hyperplasia as 5+. Hypoplastic or aplastic states are indicated by 3+, 2+, 1+, and 0. In the irradiated mice treated with isologous bone marrow and in the untreated irradiated mice, the amount and time relation of blood-cell formation in the red pulp followed the expected pattern. Blood-cell-forming tissue in the red pulp of the mice treated with rat bone marrow showed the same rapid growth of blood-cell-forming tissue (fig. 5) as seen in the bone marrow and had an equally rapid, nearly total destruction. In the spleen, however, the destroyed blood-cell-forming tissue was replaced throughout the red pulp by massive reticuloendothelial-cell proliferation of an epitheloid type. The relative status of this tissue is indicated in text-figure 2 and shown histologically in figures 6 and 7. Some hemosiderosis and erythrophagocytosis was present in the red pulp of spleens containing reticuloendothelial cells. Between days 6 and 13 this cell type tended to lose its reticuloendothelial character by having a discrete cell membrane. At the later intervals, it seemed to be only a hemosiderin-laden macrophage or an erythrophagocytic cell. Some of these cells having characteristics of a reticuloendo-


DEGREE OF BLOOD CELL FORMING TISSUE IN RED PULP OF SPLEEN
+++++
++++
+++
++
+
NORMAL DEGREE OF BLOOD CELL FORMING TISSUE IN GROUP 4 MICE
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME AFTER IRRADIATION (days)

TEXT-FIGURE 2.—Effect of irradiation and bone-marrow injection on degree of blood-forming tissue in red pulp of spleen of individual serially sacrificed (C3H × 101)F_1 mice. ■ 710 r + rat bone marrow; ○ 710 r + isologous bone marrow; ● 710 r only; □ animal died after 710 r + rat bone marrow; △ relative status of reticuloendothelial-cell replacement of red pulp in sacrificed mice given 710 r + rat bone marrow; ▲ relative status of reticuloendothelial-cell replacement in mice found dead after 710 r + rat bone marrow.


DEGREE OF LYMPHOCYTE FORMATION IN WHITE PULP OF SPLEEN
++++
+++
++
+
NORMAL DEGREE OF LYMPHOCYTE FORMATION IN WHITE PULP OF SPLEEN OF GROUP 4 MICE
BEGINNING OF GERMINAL CENTER FORMATION
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME AFTER IRRADIATION (days)

TEXT-FIGURE 3.—Effect of irradiation and bone-marrow injection on degree of lymphocyte formation in white pulp of spleen of individual serially sacrificed (C3H $\times$101)F_1 mice. ■ 710 r + rat bone marrow; ○ 710 r + isologous bone marrow; ● 710 r only; □ animal died after 710 r + rat bone marrow.

thelial cell or macrophage were also seen in the destroyed bone marrow, and many were present in lymph nodes.

The status of the white pulp of the spleen in the 4 groups of mice is shown in text-figure 3. In irradiated mice injected with isologous bone marrow, recovery of the white pulp was faster than in untreated mice. Irradiated animals injected with rat bone marrow did not live long enough to show recovery of white pulp. In the animals treated with isologous bone marrow, recovery of bone marrow and splenic red pulp preceded regeneration of white pulp.

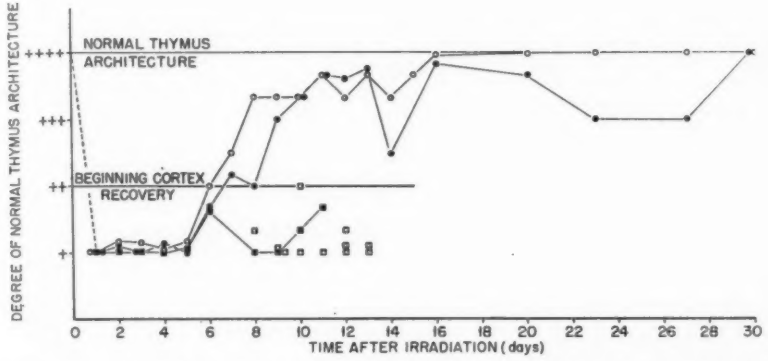
Thymus

The degree of recovery of normal histologic architecture and, to some extent, the size of the thymus as judged by histologic sections were relatively independent of bone-marrow treatment except in rat bone marrow, where no significant recovery occurred. In these very sick mice, recovery might not be expected. The results given in text-figure 4 show 4+ as the normal histologic character of the thymus. Thymus weights were not taken. In the mice treated with isologous bone marrow, recovery of bone marrow and splenic red pulp preceded regeneration of thymus.

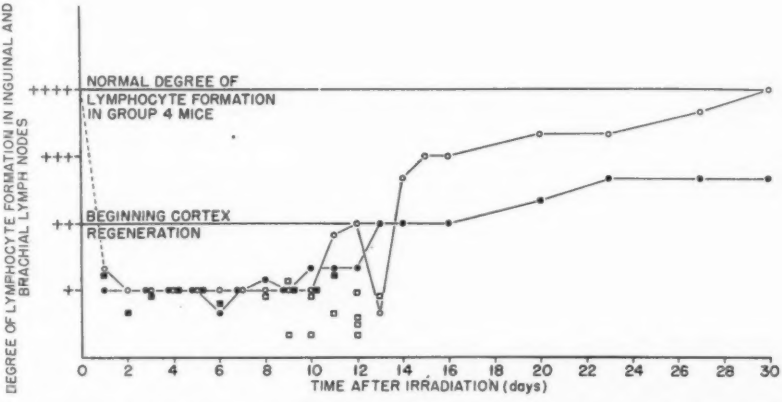
Lymph Nodes

Recovery of lymphopoiesis in the inguinal and brachial lymph nodes is shown in text-figure 5. Normal histologic architecture was recorded as 4+. The mice treated with isologous bone marrow recovered more rapidly than the untreated, irradiated mice. No significant recovery occurred in the mice treated with rat bone marrow. Reticuloendothelial cells of the type seen in the spleen appeared in the lymph nodes about the 6th day after irradiation. Some hemosiderin formation and erythrophagocytosis

was also present. A marked degree of plasma-cell formation was observed in a few lymph nodes. In the mice treated with isologous bone marrow and in the untreated, irradiated mice, recovery of marrow preceded regeneration of lymph nodes.



TEXT-FIGURE 4.—Effect of irradiation and bone-marrow injection on degree of normal thymus architecture of individual serially sacrificed (C3H × 101)F_1 mice. ■ 710 r + rat bone marrow; ○ 710 r + isologous bone marrow; ● 710 r only; □ animal died after 710 r + rat bone marrow.


DEGREE OF LYMPHOCYTE FORMATION IN INGUINAL AND BRACHIAL LYMPH NODES
++++
+++
++
+
NORMAL DEGREE OF LYMPHOCYTE FORMATION IN GROUP 4 MICE
BEGINNING CORTEX REGENERATION
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME AFTER IRRADIATION (days)

TEXT-FIGURE 5.—Effects of irradiation and bone-marrow injection on degree of lymphocyte formation in inguinal and brachial lymph nodes of individual serially sacrificed (C3H × 101)F_1 mice. ■ 710 r + rat bone marrow; ○ 710 r + isologous bone marrow; ● 710 r only; □ animal died after 710 r + rat bone marrow.

Cause of Death in 710 r Mice Given Rat Bone Marrow

In 12 animals autopsied on days 8 to 13, 10 showed hemorrhagic areas in subcutaneous tissues, intestines, or other organs. Many showed pale, anemic-appearing tissues and had thin, watery blood. Four showed bacterial infection of the nonpurulent type in various tissues. The death

of these animals was attributed primarily to the secondary bone-marrow failure and the immunologic disease process that produced it.

Evidence for Transplantation and Growth of Rat Bone Marrow in Irradiated Mice

Imprints from fresh spleen and bone marrow were made from selected mice in all groups (*4*). These imprints, when stained by the alkaline-phosphatase method, showed granulocytes of the rat type in both groups of mice injected with rat bone marrow. In the irradiated group receiving rat bone marrow, there were large numbers of alkaline phosphatase-positive cells during the period of hematopoietic proliferation, indicating that the new granulopoiesis was rat tissue. With the destruction of blood-cell-forming tissue in the animals of group 1, the alkaline phosphatase-positive cells were greatly reduced in the imprints of bone marrow and spleen. In the nonirradiated mice given rat bone marrow, the alkaline phosphatase-positive cells disappeared in a few days.

In none of the mice treated with rat bone marrow was it possible to demonstrate rat red blood cells in the peripheral blood. Makinodan's technique (*3*) was used.

Discussion and Conclusions

The 100 percent mortality in mice exposed to 710 r of total-body radiation and then injected with rat bone marrow was caused by a secondary degeneration of the transplanted bone marrow. The immediate cause of death was the associated hemorrhage, infection, and anemia.

We checked this observation by giving isologous bone marrow to the mice at an appropriate interval after the 710 r exposure and the injection of rat bone marrow. In a separate experiment, 20 (C3H $\times$ 101) F_1 mice were exposed to 710 r and given rat bone marrow intravenously. Six days later they became quite sick, and each mouse was given about 80 $\times$ 10^6 isologous bone-marrow cells intravenously. Only 4 mice died during the ensuing 16 days; the other 16 recovered.

The original experiment with rat bone marrow and a dose of 710 r was undertaken to test the hypothesis that late deaths (after more than 30 days) in mice receiving 950 r and rat bone marrow were caused by recovery of the radiation-damaged host animal's immune mechanism (*4*). The recovery resulted in a host-antibody reaction to the transplanted antigen. We reasoned that a lower dose of radiation (710 r) would still result in successful transplantation of the rat bone marrow and that a reduced injury to the host's immune response would allow its quicker recovery and violent immune response would occur. This expectation was borne out by the experiments.

In the red pulp of the spleen, the destruction of blood-cell-forming tissue was associated with massive proliferation of reticuloendothelial cells. To a lesser extent, these cells were also seen in the lymph nodes and bone marrow. Presumably, they represented the cellular component of the

immune response to rat bone marrow. However, the relation of these cells to erythrophagocytosis and hemosiderin formation was also apparent.

This unusual proliferation of reticuloendothelial cells of the epitheloid type is a unique tissue alteration. A somewhat similar change was seen at autopsy in lethally irradiated mice that were treated with dog bone marrow (Congdon, unpublished data).

Trentin (*5*) has reported that all CBA mice exposed to radiation in the midlethal dose range (550 r) died in 15 days when injected with bone marrow from a (Cb $\times$ CBA)F_1 hybrid. This exposure without bone-marrow injection caused 12 percent 30-day mortality. At higher radiation exposures (770 r), the CBA mouse receiving (Cb $\times$ CBA)F_1 blood-forming tissue showed good 30-day survival, whereas the X-ray controls all died in 30 days. The latter type of observation was made by several groups (*6–8*). Presumably, the mechanism of death in Trentin's experiment in the midlethal dose range is basically the same as that reported in the present work with (C3H $\times$ 101)F_1 mice exposed to midlethal X radiation and injected with rat bone marrow.

The recovery of lymphatic tissues in the mice treated with isologous bone marrow is of interest. Recovery of bone marrow and red pulp in the spleen of these mice preceded that of the thymus, lymph nodes, and white pulp of the spleen. This sequence of events suggests that recovery of lymphatic tissues is dependent on the animal's state of health rather than directly on the injected bone marrow. In the mice that received 710 r only, the recovery of lymphatic tissues also tended to follow bone-marrow recovery. Relatively insignificant normal lymphatic-tissue recovery occurred in the irradiated mice injected with rat bone marrow. Since these animals were quite sick, failure of lymphatic tissue to regenerate might, in part, be caused by a stress reaction.

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(*5*) Trentin, J. J.: Effect of X-ray dose on mortality and skin transplantability in mice receiving F_1 hybrid marrow. Proc. Soc. Exper. Biol. & Med. 93: 98–100, 1956.

(*6*) Lorenz, E., Congdon, C., and Uphoff, D.: Modification of acute irradiation injury in mice and guinea-pigs by bone marrow injections. Radiology 58: 863–877, 1952.

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(*8*) Trentin, J. J.: Mortality and skin transplantability in X-irradiated mice receiving isologous, homologous, or heterologous bone marrow. Proc. Soc. Exper. Biol. & Med. 92: 688–693, 1956.

PLATE 41

FIGURE 1.—Nearly normal femoral bone-marrow cellularity in a mouse sacrificed 6 days after 710 r and intravenous injection of rat bone marrow. Hematoxylin and eosin. × 250

FIGURE 2.—Nearly normal femoral bone-marrow cellularity in a mouse sacrificed 6 days after 710 r and intravenous injection of isologous bone marrow. Hematoxylin and eosin. × 250

FIGURE 3.—Marked reduction in femoral bone-marrow cellularity of a mouse sacrificed 6 days after exposure to 710 r. Hematoxylin and eosin. × 250

FIGURE 4.—Marked secondary reduction in femoral bone-marrow cellularity of a mouse sacrificed 9 days after 710 r and intravenous injection of rat bone marrow. Hematoxylin and eosin. × 250

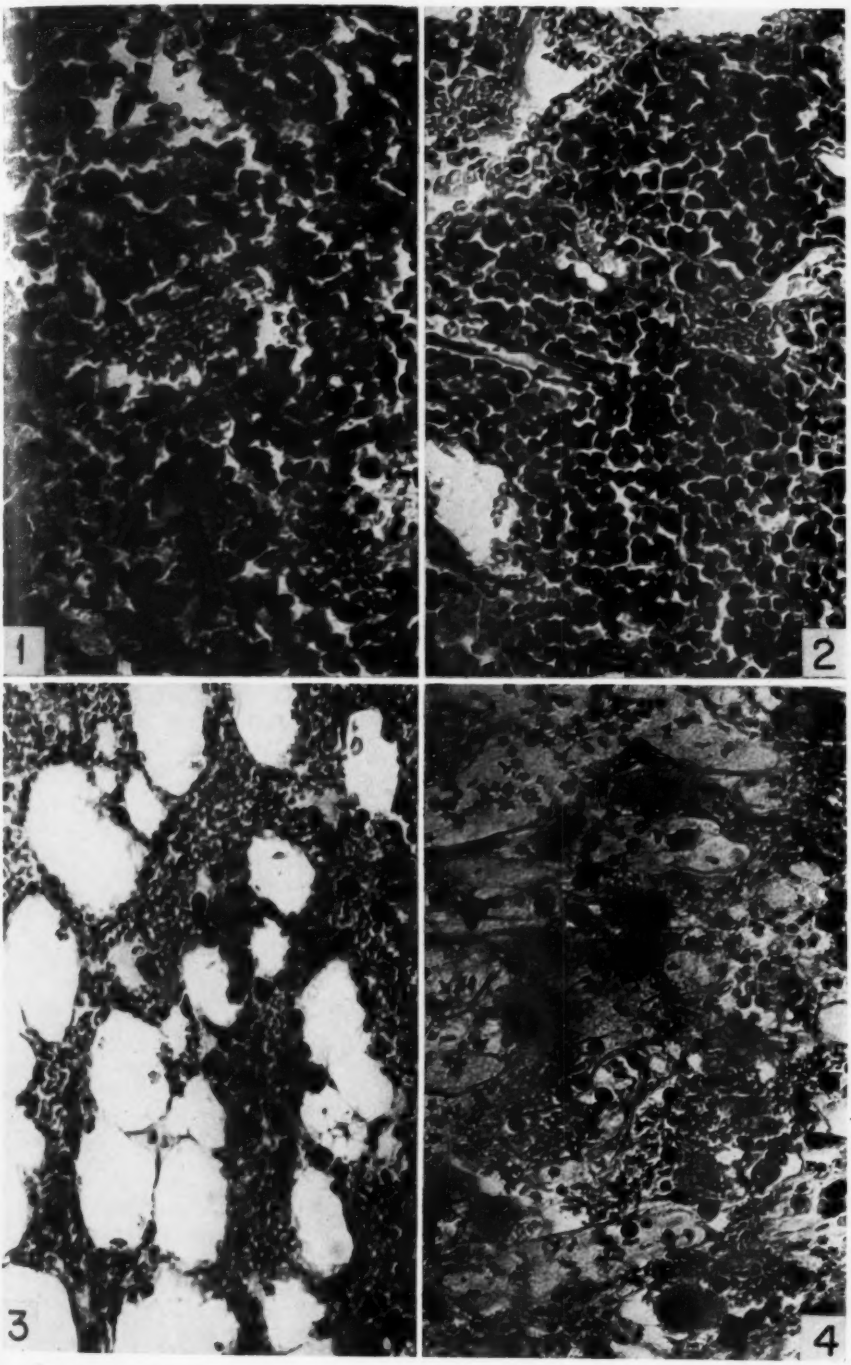

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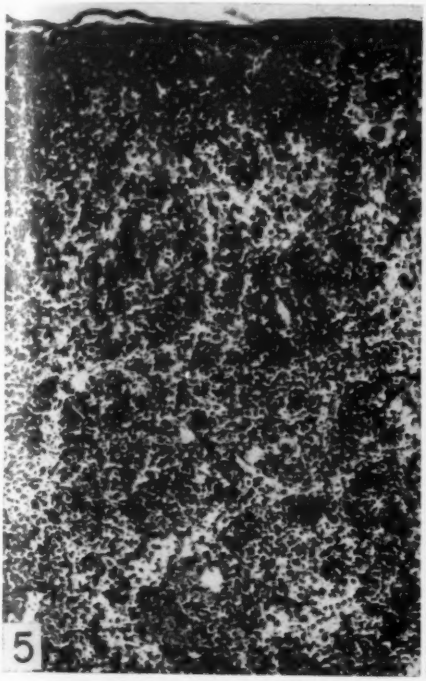
PLATE 42

FIGURE 5.—Hyperplasia of the red pulp of the spleen of a mouse sacrificed 5 days after 710 r and intravenous injection of rat bone marrow. Hematoxylin and eosin. × 145

FIGURE 6.—Nearly total destruction of blood-forming elements in the red pulp of the spleen of a mouse sacrificed 6 days after 710 r and intravenous injection of rat bone marrow. Reticuloendothelial-cell proliferation of the epitheloid-cell type fills the red pulp. A splenic nodule can be seen in the *lower left* of the photograph. Hematoxylin and eosin. × 145

FIGURE 7.—Higher-power view of cells shown in figure 6. Many hemosiderin granules are present. Hematoxylin and eosin. × 250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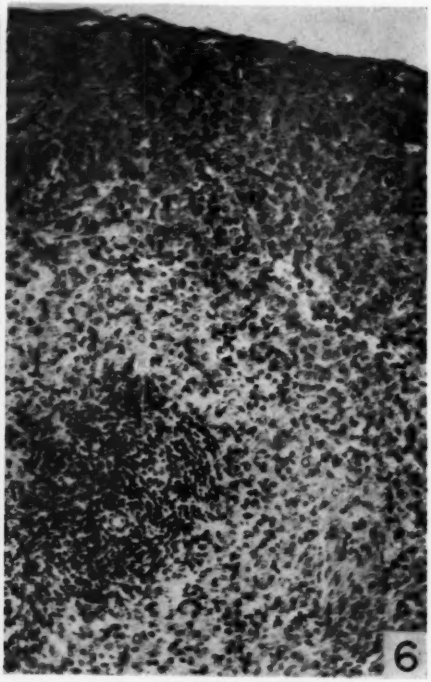
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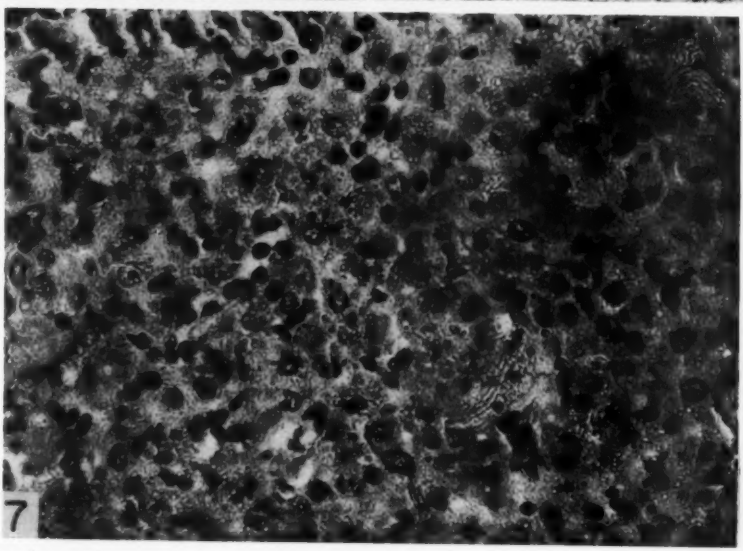
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Delayed Effects in Mice Following Acute Total-Body X Irradiation: Modification by Experimental Treatment [1, 2]

JOANNE HOLLCROFT, EGON LORENZ,[3] ELIZA MILLER, CHARLES C. CONGDON,[4] ROBERT SCHWEISTHAL,[5] and DELTA UPHOFF,[6] *Radiation Branch, National Cancer Institute,[7] Bethesda, Maryland; Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee; and Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Illinois*

Much work has been done on experimental modification of the 30-day survival of animals receiving acute lethal total-body radiation. Comparatively few data have been available on the delayed effects in animals exposed to sublethal doses of X radiation or lethal doses of X radiation rendered sublethal by spleen shielding or administration of protective chemicals.

The present study was made to evaluate the delayed effects when irradiation was modified by spleen shielding or chemical protection or when exposure was given at birth.

Experimental Method

Six groups of C3Hf/He mice were used in these studies. Group I consisted of mice set aside as unirradiated controls during the period June to December 1948. In group II, mice were exposed to a single dose of 400 r within 12 hours of birth. The exposures were given at a dose rate of 61 r per minute as measured in air. The 180 kvp 20 ma. beam was filtered with 0.25 mm. Cu and 0.51 mm. Al, and the distance from target to the middle of the mouse was 50 cm. The treatments took place as mice became available from August 1948 to May 1949.

At approximately 3 months of age, the mice in groups III, IV, and V were anesthetized with pentobarbital sodium;[8] the spleens were exteriorized and the mice were irradiated at a distance of 54 cm. from the targets of 2 opposing tubes. The two 180 kvp 20 ma. beams were filtered with 1.06 mm. Al and 0.25 mm. Cu. The dose rate was 93 r per minute. In

[1] Received for publication November 29, 1956.

[2] This work performed in part under the auspices of the U. S. Atomic Energy Commission. A portion of the data was analyzed at Oak Ridge under AEC Contract No. W-7405-eng-26.

[3] Deceased February 12, 1954. The experiments reported here were planned by Dr. Lorenz and nearly completed prior to his death. The analysis of the data and preparation of the manuscript were completed by the other workers.

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[5] *Present address:* Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill.

[6] The authors wish to thank Dr. Leon O. Jacobson and coworkers from the Argonne Cancer Research Hospital, University of Chicago, for assisting with the spleen shielding; Bruce Weed and Marion Matthews for technical assistance; and Henry Meyer for the irradiations.

[7] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[8] Nembutal, Abbott.

group III, the spleens of the mice were protected by lead shields during the single whole-body exposure to a sublethal dose of 400 r. The spleens of group IV mice were sham-protected during exposure to a single dose of 400 r. In group V, the spleens of the mice were protected with lead shields while a single LD100 of 900 r was administered. The technique of spleen shielding followed that described by Jacobson (*1*). These 3 experiments were performed during the period January to June, 1950.

At about 3 months of age, the mice in group VI were given intravenous injections of 950 mg. per kg. cysteine (adjusted to *p*H 7) 15 minutes prior to a single dose of 900 r. The physical conditions for X irradiation were the same as in group V. Two minutes prior to irradiation cysteine-injected mice, in groups of 8, were placed in a lucite box through which a mixture of 94 percent helium plus 6 percent oxygen flowed at a rate of 1 l. per minute; they were kept under these conditions throughout the irradiation exposure. This experiment was performed during the period March to May, 1953.

The 496 males and 475 females reported in this paper were the 30-day survivors from the above groups.

After irradiation, the animals were kept in plastic cages and given tap water and Purina chow. The body weights were taken periodically and blood counts were made at intervals on the two 900 r groups. Although some animals were killed when they became moribund, most were allowed to live out their life span. Autopsy examinations were made on nearly all animals and the tissues were fixed in Zenker-formol solution.

For the purposes of this study it was important to determine the survival time of mice following a given procedure. In a number of mice found dead autolysis obscured finer histologic details. Nevertheless, the pathologic studies indicated important differences of response among the 6 groups and offered some explanation for the variations in survival time.

Results

Survival Time

The unirradiated control females lived significantly longer than the unirradiated control males (table 1). The reverse was seen in the 900 r group protected by cysteine and anoxia, *i.e.*, the males lived significantly longer than the females. No such sex difference in survival time was noted among the other groups.

Irradiation shortened the life span of all groups. The reduction of life expectancy per roentgen, as defined by Sacher (*2*), was higher for the animals irradiated at birth and very much lower for males protected against 900 r with cysteine and anoxia. There was no difference in survival between the 400 r spleen-shielded and 400 r sham spleen-shielded groups. For females the reduction of life expectancy per r was higher than for males.

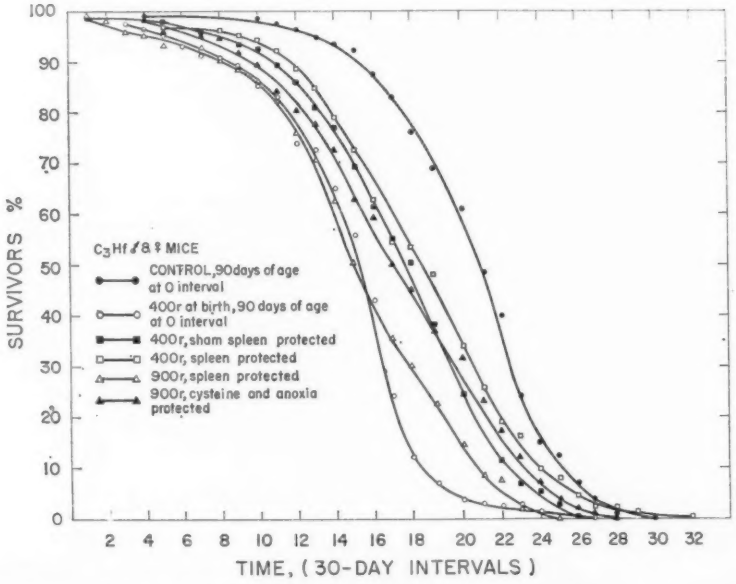
When the male and female survival data were combined for simplicity (text-fig. 1), the survival curves of the cysteine+anoxia group followed

TABLE 1.—*Survival of C3Hf mice after acute doses of X radiation*

Group	Experimental conditions	X-ray dose (*r*)	Number of mice	Age at start (*days*)	Mean survival time ± S.E. (*days*)	Reduction in life span† (*percent*)	K:* Reduction of life expectancy per r ($\times 10^4$)
			Males				
I	Control	0	104	0	671 ± 11.1	—	—
II	Birth	400	97	0	533 ± 13.9	20.7	5.17
III	Spleen protected	400	71	87.7	543 ± 18.8	6.9	1.73
IV	Sham spleen-protected	400	79	92.2	525 ± 14.8	9.3	2.33
V	Spleen protected	900	64	91.8	465 ± 20.2	19.7	2.18
VI	Cysteine + anoxia	900	81	97.5	544 ± 16.6	5.1	0.58
			Females				
I	Control	0	91	0	737 ± 12.3	—	—
II	Birth	400	88	0	508 ± 12.6	31.1	7.77
III	Spleen protected	400	74	86.9	532 ± 17.4	18.3	4.57
IV	Sham spleen-protected	400	75	90.1	493 ± 16.8	23.8	5.95
V	Spleen protected	900	67	90.2	433 ± 16.5	33.1	3.67
VI	Cysteine + anoxia	900	80	98.0	484 ± 14.2	24.3	2.7

*$K=\frac{Tc-T}{DTc}$, where D is dose.

†$\frac{Tc-T}{Tc}$, where Tc is the survival time of the controls corrected for the age of experimental animals at treatment; T is the survival time of the experimental animals.



TEXT-FIGURE 1.—Survival curves of C3Hf mice after X irradiation.

those of the mice irradiated with 400 r at 3 months of age. The curves of the 900 r spleen-protected animals, showing a shorter life span, followed the curve of the 400 r-at-birth group.

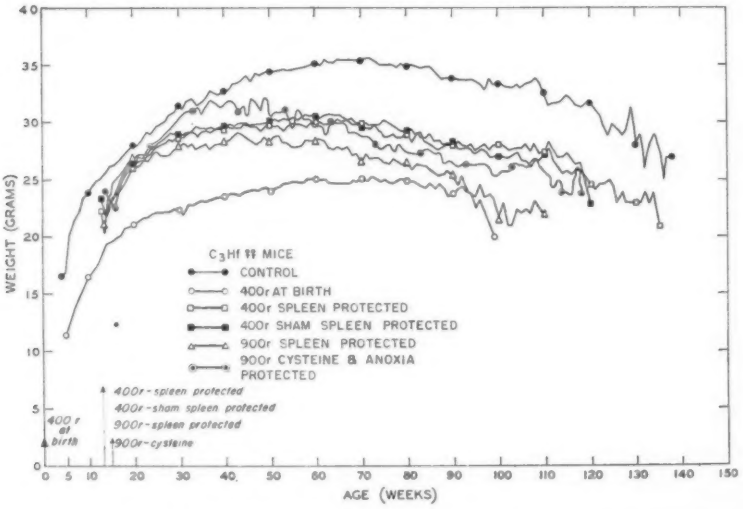
Body Weight

The weight curves (text-figs. 2 and 3) showed that irradiation prevented the mice from attaining the maximum weight of the controls. Irradiation with 400 r at birth stunted the growth of both males and females, and the weight curves were well below those of the other groups.

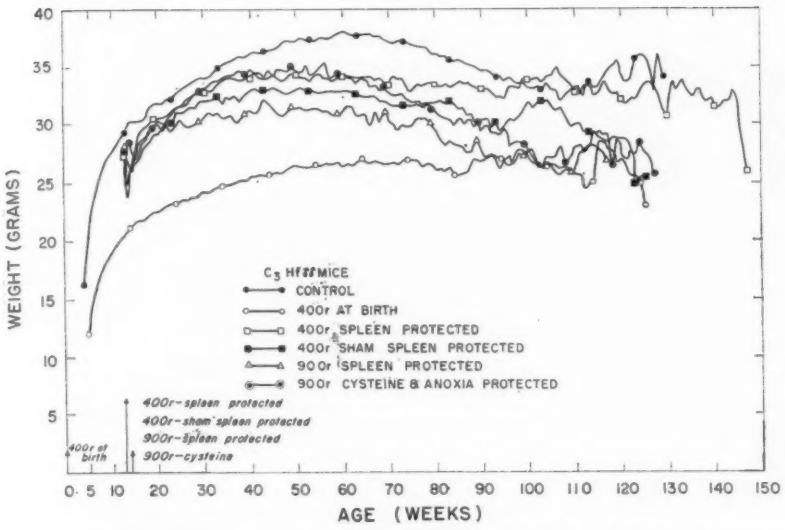
Following the initial drop after irradiation, the weight curves of the mice irradiated with 900 r at 3 months of age rose, paralleling the unirradiated controls for about 12 weeks when the spleen-protected animals stopped gaining. In general, the weights of the other male mice stopped increasing about 23 weeks after irradiation, whereas the female weights leveled at 18 weeks. An exception occurred when female animals treated with cysteine plus anoxia continued to gain weight for 23 weeks. This group also differed from the others in that their weights started dropping at 37 weeks, while those of most other groups irradiated at maturity declined at about 57 weeks. The weights of the 400 r spleen-protected males did not show the characteristic drop in weight until the last weeks of life.

Peripheral Blood

Blood counts were done in both 900 r groups. No difference was noted in the males as opposed to the females and, therefore, the composite data were used (text-fig. 4). The level of white blood cells in the mice pro-



TEXT-FIGURE 2.—Weight curves of C3Hf female mice after X irradiation. Arrows at *lower left* indicate age of mice at treatment.



TEXT-FIGURE 3.—Weight curves of C3Hf male mice after X irradiation. Arrows at *lower left* indicate age of mice at treatment.

tected by cysteine and anoxia fell lower than did that in the spleen-protected mice following a 900 r dose. The erythrocyte counts did not differ significantly in the 2 groups (text-fig. 5).

Carcinogenesis

Irradiation was more carcinogenic for females than for males, as shown in table 2. (The number of animals given in the table was the number from which histologic sections were obtained; in some animals autolysis was too great to permit sections to be obtained.) The average number of tumors per female mouse was at least doubled by irradiation, whereas in males the average number per mouse was increased by 3 percent in the 900 r spleen-protected group and 54 percent in the 400 r spleen-protected group. Some of this sex difference can be explained by the large incidence of ovarian tumors. However, a great variety of tumors was found at autopsy, as shown in tables 3 and 4.

Hepatomas occurred more frequently in the control mice than did other neoplasms and more frequently in males than in females ($P<0.01$), except in the 400 r sham-operated and the 900 r spleen-protected groups. Irradiation did not increase hepatoma incidence. The incidence found in the male 400 r sham spleen-protected group was lower than that of the control males ($P<0.01$). The male mice protected with cysteine and anoxia had a higher percentage of hepatomas than the 900 r spleen-protected males ($P<0.01$). Multiple hepatomas occurred with about the same frequency in all groups.

TABLE 2.—*Number of neoplasms found in C3Hf mice at autopsy*

Group	Experimental conditions	X-ray dose (*r*)	No. autopsied*	Average age at death (*mos.*)	No. mice with following no. primary tumors: 0	1	2	3	4	Total number with tumors	Percent with tumors	Average no. primary tumors per mouse
	Males											
I	Control	0	80	22. 5	37	33	10	—	—	43	54	0. 66
II	Birth	400	78	17. 9	27	45	5	1	—	51	65	0. 74
III	Spleen protected	400	51	21. 5	12	27	11	1	—	39	76	1. 02
IV	Sham spleen-protected	400	65	20. 4	18	34	12	1	—	47	72	0. 94
V	Spleen protected	900	55	18. 7	26	21	7	1	—	29	53	0. 69
VI	Cysteine+anoxia	900	75	21. 2	18	42	13	1	1	57	76	1. 00
	Females											
I	Control	0	73	24. 0	41	19	11	1	1	32	44	0. 66
II	Birth	400	70	16. 7	2	42	23	2	1	68	97	1. 01
III	Spleen protected	400	62	20. 9	8	24	19	9	2	54	87	1. 56
IV	Sham spleen-protected	400	65	19. 4	3	25	25	11	1	62	95	1. 72
V	Spleen protected	900	56	17. 6	13	23	16	3	1	43	77	1. 21
VI	Cysteine+anoxia	900	61	20. 3	9	29	18	4	1	52	85	1. 32

*On which histologic sections were obtained.

TABLE 3.—*Neoplasms found in male C3Hf mice at autopsy*

Group	Experimental conditions	Mice with following types of tumor:*†													
		Adrenal		Reticular tissue		Carcinoma		Sarcoma		Lung—primary		Hepatoma		Hemangioendothelioma	
		Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)
I	Control.....	0 (0/64)	—	0.013 (1/80)	25.0	0.050 (4/80)	22.2	0.013 (1/80)	27.0	0.113 (9/80)	23.3	0.425 (34/80)	22.8	0.050 (4/80)	23.6
II	400 r At birth...	0.013 (1/75)	18.5	0.013 (1/78)	12.0	0.026 (2/78)	20.5	0 (0/78)	—	0.090 (7/78)	17.8	0.564 (44/78)	18.2	0.026 (2/78)	20.7
III	400 r Spleen protected.	0.075 (3/40)	24.1	0.118 (6/51)	19.6	0.059 (3/51)	25.8	0.020 (1/51)	15.7	0.196 (10/51)	24.5	0.549 (28/51)	22.1	0.020 (1/51)	18.0
IV	400 r Sham spleen-protected.	0.033 (2/60)	24.3	0.215 (14/65)	18.4	0.091 (1/65)	19.0	0.015 (1/65)	24.9	0.108 (7/65)	24.4	0.215 (14/65)	21.6	0 (0/65)	—
V	900 r Spleen protected.	0.111 (5/45)	18.7	0.068 (1/55)	24.0	0.036 (2/55)	21.8	0.036 (2/55)	22.1	0.167 (9/54)	19.5	0.286 (14/49)	20.0	0.036 (2/55)	21.0
VI	900 r Cysteine +anoxia.	0.138 (8/58)	25.3	0.171 (12/70)	18.1	0.109 (8/73)	24.6	0.014 (1/73)	19.5	0.056 (4/71)	24.3	0.533 (40/75)	22.7	0 (0/75)	—

*Figures in parentheses refer to number of mice with neoplasms divided by number of mice on which sections were obtained.
†Age = average age at death.

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TABLE 4.—*Neoplasms found in female C3Hf mice at autopsy*

Group	Experimental conditions	Mice with following types of tumor:*†									
		Adrenal		Reticular tissues		Non-mammary carcinoma		Non-mammary sarcoma		Lung—primary	
		Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)
I	Control	0. 028 (2/71)	28. 9	0. 082 (6/73)	25. 9	0. 041 (3/73)	22. 4	0. 014 (1/73)	28	0. 109 (8/73)	26. 7
II	400 r At birth	0. 087 (6/69)	20. 1	0. 057 (4/70)	7. 8	0 (0/70)	—	0. 057 (4/70)	13. 8	0. 059 (4/58)	19. 1
III	400 r Spleen protected	0. 153 (9/59)	24. 1	0. 082 (5/61)	18. 9	0. 081 (5/62)	20. 5	0. 097 (6/62)	19. 9	0. 083 (5/60)	20. 5
IV	400 r Sham spleen-protected.	0. 154 (10/65)	23. 1	0. 108 (7/65)	14. 9	0. 077 (5/65)	22. 0	0. 046 (3/65)	17. 8	0. 123 (8/65)	21. 6
V	900 r Spleen protected	0. 192 (10/52)	18. 5	0. 018 (1/56)	16. 0	0. 161 (9/56)	16. 8	0. 125 (7/56)	14. 5	0. 074 (4/54)	18. 3
VI	900 r Cysteine+anoxia	0. 208 (10/48)	23. 2	0. 117 (7/60)	17. 8	0. 083 (5/60	22. 2	0. 083 (5/60)	21. 1	0. 070 (4/57)	20. 7

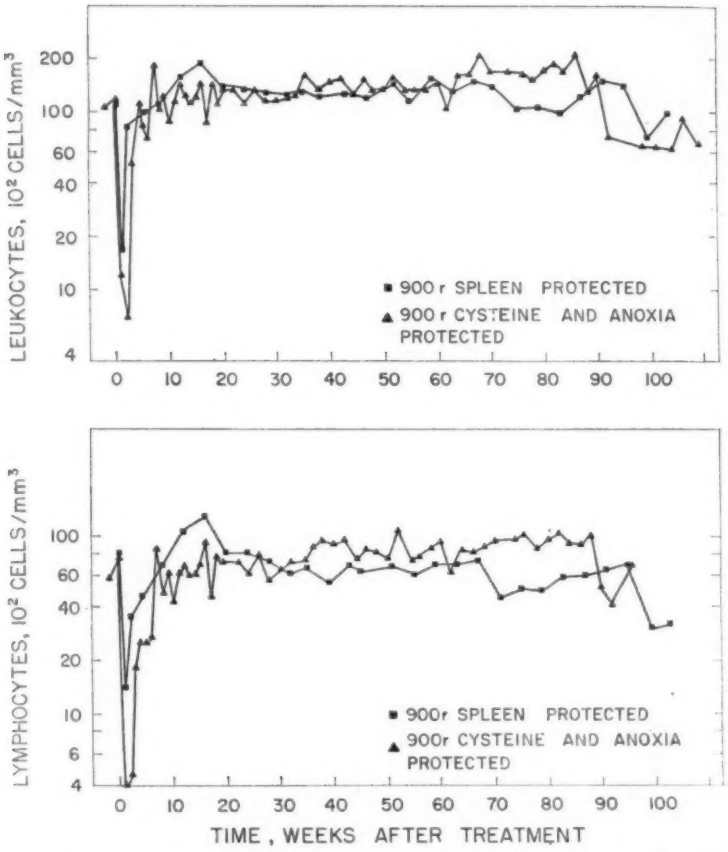
TABLE 4.—*Neoplasms found in female C3Hf mice at autopsy*—Continued

Group	Experimental conditions	Mice with following types of tumor: *†—Continued							
		Hepatoma		Hemangioendothelioma		Ovarian		Mammary	
		Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)
I	Control	0. 178 (13/73)	24. 3	0 (0/73)	—	0. 109 (8/73)	24. 2	0. 096 (7/73)	24. 0
II	400 r At birth	0. 203 (14/69)	18. 4	0. 029 (2/70)	20. 0	0. 914 (64/70)	17. 2	0. 014 (1/70)	17. 0
III	400 r Spleen protected	0. 210 (13/62)	22. 6	0. 032 (2/62)	21. 0	0. 677 (42/62)	22. 1	0. 164 (10/61)	19. 9
IV	400 r Sham spleen-protected	0. 200 (13/65)	22. 6	0. 031 (2/65)	20. 8	0. 754 (49/65)	19. 9	0. 169 (11/65)	17. 7
V	900 r Spleen protected	0. 143 (7/49)	19. 3	0 (0/56)	—	0. 358 (19/53)	17. 3	0. 196 (11/56)	17. 3
VI	900 r Cysteine+anoxia	0. 200 (2/60)	23. 9	0 (0/60)	—	0. 483 (29/60)	21. 0	0. 133 (8/60)	22. 8

*Figures in parentheses refer to number of mice with neoplasms divided by number of mice on which sections were obtained.
†Age = average age at death.

Irradiation did not alter the incidence of lung tumors, which were the next most frequent neoplasm of the control group.

Ovarian tumors also occurred with moderate frequency in the control animals, but unlike lung tumors and hepatomas all irradiation treatments increased ovarian tumors ($P<0.01$ for all groups). Irradiation at birth was more tumorigenic to the ovaries than irradiation at maturity.



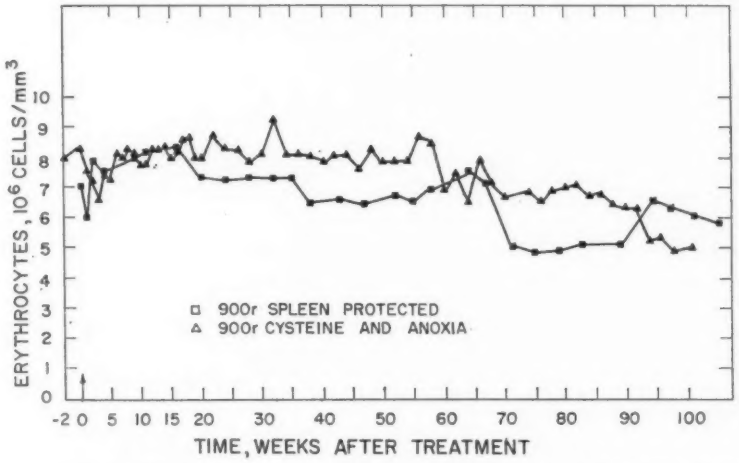
TEXT-FIGURE 4.—Leukocyte and lymphocyte counts of C3Hf mice exposed to 900 r X irradiation.

The ovarian tumors found in mice irradiated at birth were usually tubular adenomas, although a number of mixed tubular adenomas and granulosa-cell tumors were also present (table 5). Most of the ovarian tumors were bilateral. The 400 r spleen-protected females had about an equal number of tubular adenomas, granulosa-cell tumors, and mixed tubular adenomas and granulosa-cell tumors. The majority of ovarian tumors in the sham spleen-protected mice were granulosa-cell tumors.

In the mice irradiated with 900 r the granulosa-cell tumors were also the more prevalent type. The tumor type apparently was not dependent on age of the animal at death. The ovarian tumors were classified predominantly on the basis of the major component present in the tumor sectioned.

The mammary-tumor incidence in C3Hf female controls was low. Four sarcomas, 2 adenocarcinomas, as shown in table 6, and a squamous-cell carcinoma (not listed) were noted in the mammary-gland area at autopsy. Only 1 mammary tumor, a mixed adenoacanthoma and sarcoma, was seen in the mice irradiated at birth. This represented a significant ($P<0.05$) decrease in incidence.

Although there was no difference between controls and irradiated mice with respect to the occurrence of mammary sarcomas, the mam-



TEXT-FIGURE 5.—Erythrocyte counts of C3Hf mice exposed to 900 r X irradiation.

mary-adenocarcinoma incidence was increased in the two 400 r groups ($P<0.05$ for each) and the 900 r spleen-protected group ($P<0.01$). When the data for adenocarcinomas and adenoacanthomas were combined, the 900 r cysteine+anoxia treated mice also showed an increase ($P<0.05$) and the increase in the other groups became more significant.

The neoplasms of the reticular tissues found in C3Hf mice were generalized lymphocytic neoplasms, reticulum-cell sarcomas, and granulocytic leukemia, as shown in table 7. In addition, a mast-cell tumor occurred in a female of the cysteine+anoxia group. The total number of neoplasms of reticular tissues was small and in order to obtain an over-all picture of the differences among the experimental groups the male and female data were combined.

A few small sex differences should be pointed out. Four of the reticulum-cell sarcomas in the control group and 4 in the cysteine+anoxia

TABLE 5.—*Types of ovarian tumors found at autopsy*

Group	Experimental conditions	X-ray dose (*r*)	Number autopsied*	Mice with following types of tumor:									
				Tubular adenomas		Mixed tubular adenomas and granulosa-cell tumors		Granulosa-cell tumors		Luteinizing granulosa-cell tumors		Luteomas	
				No.	Age† (*mos.*)	No.	Age† (*mos.*)	No.	Age† (*mos.*)	No.	Age† (*mos.*)	No.	Age† (*mos.*)
I	Control	0	73	0	—	1	24. 5	6	24. 7	1	20. 5	0	—
II	Birth	400	70	49	16. 9	12	17. 6	1	16. 8	1	17. 5	1	21. 0
III	Spleen protected	400	62	19	21. 5	10	25. 8	13	20. 8	0	—	0	—
IV	Sham spleen-protected	400	65	9	18. 8	9	21. 1	29	20. 2	2	21. 8	0	—
V	Spleen protected	900	53	6	18. 3	1	14. 5	11	17. 1	1	17. 0	0	—
VI	Cysteine+anoxia	900	60	5	24. 5	2	25. 7	22	19. 7	0	—	0	—

*On which sections were obtained.
†Age = average age at death.

TABLE 6.—*Mammary tumors found at autopsy*

Group	Experimental conditions	X-ray dose (*r*)	Number autopsied*	Mice with following types of tumor:					
				Sarcoma		Adenocarcinoma		Adenoacanthoma	
				No.	Age† (*mos.*)	No.	Age† (*mos.*)	No.	Age† (*mos.*)
I	Control	0	73	4	25. 0	2‡	21. 0	0	—
II	Birth	400	70	0	—	0	—	1§	17. 0
III	Spleen protected	400	61	1	21. 7	9	19. 7	0	—
IV	Sham spleen-protected	400	65	1	22. 3	9	17. 6	1	14. 5
V	Spleen protected	900	56	1	17. 0	8	18. 7	2	11. 5
VI	Cysteine+anoxia	900	60	1	24. 5	5	22. 3	2	22. 7

*On which histologic sections were obtained.
†Average age at death.
‡Also 1 squamous-cell carcinoma at 24 months of age.
§Mixed adenoacanthoma and sarcoma.

TABLE 7.—*Types of tumors of reticular tissues found in C3Hf mice at autopsy*

Group	Experimental conditions	X-ray dose (*r*)	Number autopsied*	Mice with following types of tumor:					
				Generalized lymphosarcoma		Granulocytic leukemia		Reticulum-cell sarcoma	
				No.	Age† (*mos.*)	No.	Age† (*mos.*)	No.	Age† (*mos.*)
I	Control	0	153	2	22. 7	0	—	5	26. 8
II	Birth	400	148	5	11. 0	0	—	0	—
III	Spleen protected	400	112	5	21. 4	4	14. 5	2	27. 7
IV	Sham spleen-protected	400	130	9	18. 5	10	16. 5	2	23. 0
V	Spleen protected	900	111	2	17. 5	0	—	0	—
VI	Cysteine+anoxia	900	130	10	18. 1	4	18. 4	5	17. 5

*On which sections were obtained.
†Average age at death.

group occurred in females. Of the 10 lymphosarcomas in the cysteine+anoxia group, 2 were found in the females and 8 in the males. Irradiation increased the number of lymphosarcomas in the sham spleen-protected and in the cysteine-treated males but not in the females. On the other hand, the percentage of granulocytic leukemias in both females and males of the 400 r sham spleen-protected group was greater than that of the controls.

When the data for males and females were combined and the irradiated mice compared with controls, the lymphosarcoma incidence was increased in the 400 r sham spleen-shielded animals ($P<0.01$) and in the 900 r cysteine+anoxia group ($P<0.01$). Granulocytic leukemia was also increased significantly in these 2 groups, as well as in the 400 r spleen-protected group.

In all groups the lymphosarcomas appeared to originate more frequently in the lymph nodes and spleen than in the thymus, although the thymus

TABLE 8.—*Types of adrenal tumors found in C3Hf mice at autopsy*

Group	Experimental conditions	X-ray dose (*r*)	Number autopsied*	Mice with medullary tumors		Mice with cortical tumors	
				No.	Age† (*mos.*)	No.	Age† (*mos.*)
I	Control	0	135	2	28. 9	0	—
II	Birth‡	400	144	4	20. 3	2	19. 0
III	Spleen protected	400	99	10	23. 0	2	27. 0
IV	Sham spleen-protected	400	125	8	23. 2	4	23. 0
V	Spleen protected‡	900	97	7	18. 6	7	17. 9
VI	Cysteine+anoxia	900	106	14	24. 2	4	24. 0

*On which histologic sections were obtained.
†Average age at death.
‡An additional tumor not determined.

was also involved in about 50 percent of the cases. The granulocytic leukemias showed a widespread distribution of neoplastic cells in the spleen, liver, lymph nodes, and bone marrow. This neoplasm was distinguished from leukemoid reaction by morphologic features and the fact that there were no primary conditions for the production of a granulocytosis. Other tumors were found rarely, and there was no evidence of infection in animals with this neoplasm. Unfortunately, no transplanta-

TABLE 9.—*Non-mammary sarcomas*

Group	Experimental conditions	X-ray dose (*r*)	Number autopsied*	Mice with following types of sarcoma:		
				Spindle-cell sarcoma		
				No.	Site†	Age‡ (*mos.*)
I	Control	0	73	1	Front of vertebra	20. 0
II	Birth	400	70	3	Mediastinum	6. 5
					Tail	15. 7
					Leg	20. 3
III	Spleen protected	400	62	2	Pleomorphic thoracic	17. 5
					Subcutaneous flank	22. 5
IV	Sham spleen-protected	400	65	3	Tail	14. 5
					Pelvis	17. 0
					Leg	22. 0
V	Spleen protected	900	56	3	Pelvis	15. 0
					Lumbar	17. 0
					Lymph node	17. 5
VI	Cysteine+anoxia	900	60	3	Vertebra	25. 0
					Rib	25. 0
					Abdomen	26. 5

*On which sections were obtained.
†Area of main tumor mass.
‡Age at death.

tion tests were made, and *post-mortem* autolysis was present in most mice suspected to have granulocytic leukemia.

X irradiation increased adrenal tumors except in the animals irradiated at birth. In this group and in the sham spleen-protected group the females had more adrenal tumors than the males. Most were of medullary origin, as shown in table 8. A neuroblastoma was observed in a control female.

The cysteine-protected females had more subcutaneous sarcomas than did the controls. It is possible that 1 subcutaneous spindle-cell sarcoma in the 400 r spleen-protected group was actually a mammary tumor, although the sections showed no mammary-gland elements. Most of the sarcomas in this group were osteogenic, as indicated in table 9. One mouse in the cysteine+anoxia group had an osteogenic sarcoma in the thigh, as well as a spindle-cell sarcoma surrounding the ribs.

Subcutaneous sarcomas were infrequent in the males, and no osteogenic sarcomas were found.

Non-mammary carcinomas were found more frequently in the spleen-shielded females irradiated with 900 r than in males of the same group. Irradiation increased the incidence of carcinomas in these females ($P<0.05$) but in no other animals. However, adenocarcinomas of the Harderian gland and of the kidney were found in the mice irradiated at

found in C3Hf females at autopsy

Mice with following types of sarcoma—Continued								
Osteosarcoma			Rhabdomyosarcoma			Leiomyosarcoma		
No.	Site†	Age‡ (*mos.*)	No.	Site†	Age‡ (*mos.*)	No.	Site†	Age‡ (*mos.*)
0			0			0		
1	Vertebra	12. 7	0			0		
4	Sternum Vertebra Sternum Sternum	14. 7 17. 5 22. 3 22. 3						
0			0			0		
1	Knee	22. 5	1	Subcutaneous in thoracic area	14	2	Uterus Stomach	17. 7 20. 5
2	Rib Thigh	20. 0 25. 0	1	Leg	21. 7	0		

maturity but not in the controls (*see* table 9). A number of Harderian-gland tumors were probably missed because they were not looked for routinely, except in the cysteine+anoxia protected mice. Other miscellaneous carcinomas, shown in table 10, may have been correlated with irradiation.

Other Lesions

In addition to the neoplasms, certain inflammatory and/or reactive lesions were noted at autopsy (*see* tables 11 and 12). Perhaps the most striking change produced by irradiation was the glomerulosclerosis found in all mice irradiated with 900 r. Cysteine appeared to afford some protection to the kidneys in the females, for the difference between the female groups is significant at less than a 0.01 level. Although slight glomerulosclerosis was sometimes seen in the mice irradiated with 400 r at maturity it was never as marked as that seen in the mice irradiated with 900 r.

Pyelonephritis was prevalent in the control male mice and less frequent in the irradiated mice. In the longer-lived cysteine+anoxia group the reduction in pyelonephritis was striking—the incidence was decreased by a factor of 10. It was found in only 9.6 percent of the female controls and about as frequently in females irradiated with 400 r at maturity but less so in the other groups. Amyloid was sometimes present in mice with pyelonephritis not only in the kidney but also in the spleen, liver, and adrenals.

No inflammatory kidney lesions were found in females irradiated at birth. However, about a third of these mice had cystic kidneys, as did about one half the male mice irradiated at birth. It is possible that nephrons not completely formed at birth were destroyed by the irradiation process.

In addition, irradiation at birth increased the number of male and female mice dying with acute and subacute endocarditis and thrombosis ($P<0.05$ and $P<0.01$, respectively). On the other hand, the males receiving 900 r with cysteine and anoxia protection had fewer of the heart conditions than did control males.

Not only was focal myelofibrosis in the sternal marrow more prevalent in the females than the males of the control groups, but also irradiation increased still further the incidence of this lesion in the females although not in the males. Sections of the sternal marrow from mice irradiated at birth were not reviewed and therefore data for myelofibrosis were not recorded.

Another pathologic change was seen in the livers of irradiated females bearing granulosa-cell tumors, referred to as reactive liver change in table 11. The hepatic cells appeared generally larger and more uniform in size than in livers of normal mice. Connective tissue about portal veins and bile ducts was slightly increased. Pigment-laden cells were frequent in all sections and were more numerous about portal veins. The pigment appeared to be principally hemosiderin. Small lymphocytes also accumulated in the same areas.

TABLE 10.—*Non-mammary carcinomas found in C3Hf mice at autopsy*

Group	Experimental conditions	X-ray dose (*r*)	Number autopsied*	Mice with following types of carcinoma:											
				Adenocarcinoma of the Harderian gland		Adenocarcinoma of the kidney		Miscellaneous adenocarcinoma			Squamous-cell carcinoma			Basal-cell carcinoma		
				No.	Age† (*mos.*)	No.	Age† (*mos.*)	No.	Site‡	Age† (*mos.*)	No.	Site‡	Age† (*mos.*)	No.	Site‡	Age† (*mos.*)
I	Control.....	0	153	0	—	0	—	2	Uterus Unknown	20. 5 20. 9	4	Skin Unknown Tail Subcutaneous	25. 5 24. 5 21. 3 21. 0	1	Leg	22. 5
II	Birth.....	400	148	0	—	0	—	1	Small bowel	18. 5	1	Unknown	25. 7	0	—	—
III	Spleen protected.	400	113	2	25. 3	2	20. 5	1	Uterus	22. 7	3	Ear Head Stomach	19. 0 21. 5 24. 0	0	—	—
IV	Sham spleen-protected.	400	130	1	18. 0	2	22. 0	1	Uterus	23. 3	2	Skin Head	19. 0 20. 7	0	—	—
V	Spleen protected.	900	111	2	20. 0	0	—	4	Pyloris Unknown Cecum Cecum	13. 3 17. 0 14. 3 14. 7	4	Ear Eyelid Jaw Vagina	18. 4 14. 0 15. 3 17. 7	1	Leg	25. 9
VI	Cysteine + anoxia.	900	133	8	25. 0	1	19. 0	1	Pancreas	24. 3	3	Stomach Conjunctiva Neck	24. 3 22. 5 16. 7	0	—	—

*On which sections were obtained.
†Average age of mice.
‡Area of main tumor mass.

TABLE 11.—*Inflammatory lesions and other changes found in male C3Hf mice*

Group	Experimental conditions	X-ray dose (*r*)	Males with following miscellaneous changes:*†							
			Glomerulosclerosis		Pyelonephritis		Myelofibrosis of the sternum		Endocarditis and thrombosis	
			Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)
I	Control	0	0 (0/78)	—	0. 44 (34/78)	22. 5	0 (0/80)	—	0. 089 (7/79)	21. 6
II	Birth	400	0 (0/77)	—	0. 065 (5/77)	18. 2	‡	‡	0. 273 (21/77)	20. 3
III	Spleen protected	400	0 (0/51)	—	0. 157 (8/51)	20. 8	0 (0/51)	—	0. 059 (3/51)	23. 5
IV	Sham spleen-protected	400	0 (0/65)	—	0. 231 (15/65)	19. 8	0 (0/65)	—	0. 094 (6/64)	23. 6
V	Spleen protected	900	0. 94 (47/50)	19. 6	0. 220 (11/50)	18. 1	0. 02 (1/50)	24. 1	0. 093 (5/54)	20. 9
VI	Cysteine+anoxia	900	0. 91 (63/69)	22. 6	0. 043 (3/69)	26. 3	0. 042 (3/72)	26. 8	0 (0/62)	—

*Figures in parentheses refer to number of mice with lesions divided by number of mice on which sections were obtained.
†Age = average age of mice.
‡Not examined.

TABLE 12.—*Inflammatory lesions and other changes found in female C3Hf mice*

Group	Experimental conditions	X-ray dose (*r*)	Females with following miscellaneous changes:*†									
			Glomerulosclerosis		Pyelonephritis		Myelofibrosis of the sternum		Endocarditis and thrombosis		Reactive liver change	
			Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)	Fraction	Age (*mos.*)
I	Control	0	0 (0/73)	—	0.096 (7/73)	23.9	0.082 (6/73)	25.8	0.104 (7/73)	26.4	0 (0/73)	—
II	Birth	400	0 (0/65)	—	0 (0/65)	—	‡	‡	0.269 (18/67)	18.3	0 (0/67)	—
III	Spleen protected	400	0 (0/62)	—	0.145 (9/62)	17.7	0.193 (12/62)	22.6	0.058 (3/52)	19.6	0.019 (1/52)	20.2
IV	Sham spleen-protected	400	0 (0/65)	—	0.138 (9/65)	16.9	0.307 (20/65)	21.4	0.031 (2/65)	19.7	0.109 (7/64)	19.4
V	Spleen protected	900	1.00 (52/52)	15.3	0.019 (1/52)	15.5	0.260 (13/50)	17.6	0.038 (2/52)	19.6	0.082 (4/49)	15.1
VI	Cysteine+anoxia	900	0.737 (42/57)	21.9	0.017 (1/57)	14.0	0.21 (12/57)	20.0	0 (0/56)	—	0.053 (3/57)	19.9

*Figures in parentheses refer to number of mice with lesions divided by number of mice on which sections were obtained.
†Age = average age of mice.
‡Not examined.

Although the eyes were not taken routinely for sections, sufficient sections were available to show that some degree of cataract formation in the lens was probably present in all irradiated groups. In addition to cloudy lenses, the animals irradiated at birth showed the changes in the retina described by Lorenz and Dunn (*3*). In all strain C3H mice examined at the National Cancer Institute the rod layer is absent from the retina of adult mice (*4*), so that the irradiation damage was superimposed on this anomalous condition.

The testes of all irradiated males showed tubular atrophy and decreased spermatogenesis. In mice irradiated at birth the testes showed focal atrophy of the germinal epithelium, which may have resulted from failure to develop. Similar changes were seen only infrequently in the controls.

Pneumonia occurred in about 10 percent of the mice in all groups.

Finally, many of the spleens which had been surgically mobilized showed infarction. The number of splenic infarcts was the same in all groups, but the degree of infarction appeared to be greater in the 900 r group.

Discussion

Although spleen shielding hastened the recovery of the hematopoietic system following irradiation it did not influence long-term survival. Once recovered, the hematopoietic system remained approximately normal. The blood dyscrasia in rats described by Bennett *et al.* (*5*) following protection by anoxia was not seen in either the spleen-shielded or the cysteine+anoxia protected mice. Furthermore, the incidence of induced leukemia was low in this strain. A similar dependence of life span on dose was noted by Lorenz (*6*) in studies on the effects of chronic irradiation, by Hursh *et al.* (*7*) in studying the life span of Wistar rats after single doses of X radiation, and by others (*8–11*) in mice of various strains.

The chemical protection by cysteine and anoxia afforded a dose-reducing effect, as measured by long-term survival, as well as the acute phase seen by Patt *et al.* (*12*). When the reduction in survival time of the chemically protected animals was compared with the average life-expectancy reduction factor of the other mice irradiated at maturity, it was noted that the effective dose to the males was reduced by about 77 percent while that of the females was reduced about 32 percent. No data are available on effective dose reduction by this amount of cysteine and anoxia, as measured by 30-day lethality, but 950 mg. per kg. of cysteine reduced the lethal effect of irradiation by about 40 percent in CF mice (*12*), and Dowdy, Bennett, and Chastain (*13*) used anoxia to increase the 30-day LD50 in rats from 600 to 1200 r. However, the maximum protection effected by different combinations of chemicals was found to be 50 percent reduction in dose by Salerno *et al.* (*14*). Although in preliminary experiments, Mayer, Patt, and Straube (*15*) found hypoxia combined with cysteine to be more than additive in protective effect, Devik found that hypoxia potentiated cysteine protection but that in larger doses the effect was less than additive (*16*).

The females appeared more sensitive to the long-term effects of radiation than did the males, since there was a greater reduction in their life expectancy. Male mice have been reported to be more sensitive to acute radiation lethality (*17,18*), but reports are contradictory (*19,20*).

Although the life span in nonirradiated mice was longer among the females, it was approximately the same in both irradiated males and females. This may be explained, at least partially, by the comparative increase in survival time of the males. The early deaths of many control males was caused by pyelonephritis and hepatoma, which were more frequent than in females. The pyelonephritis was markedly reduced by irradiation. Particularly in the cysteine+anoxia groups, the long life span can be attributed to lack of pyelonephritis. A similar decrease in pyelonephritis was seen in strain A males following 400 r X radiation (*21*). The females were not so susceptible to this inflammatory kidney lesion and, therefore, any reduction due to radiation was not so important in lengthening the average life span. The pyelonephritis found in the mouse kidney has been ascribed by some (*22,23*) to amyloidosis and obstruction of the collecting tubules of the kidney.

Glomerulosclerosis, described by Bennett *et al.* (*5*) and Furth *et al.* (*10*) and seen in the 900 r groups, was found in all mice of both sexes and, therefore, its life-shortening effect must have been the same in males and females. The failure of Upton *et al.* (*11*) to observe this lesion in RF mice exposed to doses up to 512 r was perhaps due to the dose difference rather the strain difference; the C3Hf mice irradiated with 400 r did not develop this lesion to a significant extent. Although cysteine and anoxia did not completely protect the kidney against glomerulosclerosis, as would be expected with a dose-reduction effect, the cysteine+anoxia protected females had fewer renal lesions than did the spleen-protected females irradiated with 900 r.

The fact that the females showed a greater reduction in life span following irradiation is also due in part to the fact that irradiation was more carcinogenic in the females than the males. Ovarian tumors accounted for this difference in large part. Although ovarian tumors were not often the immediate cause of death, they were probably a contributing factor in many animals.

The one difference in ovarian-tumor incidence in the groups irradiated at maturity was the lower incidence in the 900 r spleen-protected mice, which was probably a reflection of the shorter life span of this group. No difference in the other groups was to be expected because as little as 50 r has been found to be carcinogenic to the ovaries (*24*). In line with the results of Furth (*25*), the mice irradiated at birth were more sensitive to ovarian changes. The tumors were mainly tubular adenomas, indicating perhaps that the germinal epithelium from which they arise had a high activity at birth. However, neither Furth (*25*) nor Deringer (*26*) reported any unusual variations in ovarian-tumor types in mice irradiated at birth. Mice exposed to a nuclear detonation at an age of 6 to 12 weeks developed a preponderance of luteomas (*27*). C3Hf mice exposed to chronic *gamma*

radiation had a high percentage of granulosa-cell tumors (*24*). Mühlbock (*28*) observed a strain difference in the types of ovarian tumor induced by radiation.

Both myelofibrosis and osteogenic sarcomas were found only in the females. However, not all of the females had granulosa-cell tumors. Estrogen treatment has been reported to produce osteosclerosis and fibrosis (*29*).

Adrenocortical tumors have been induced in C3H (*30*) and other strains (*31*) by castration. Medullary lesions in the BRS strain following castration were reported by Smith *et al.* (*32*), who also noted medullary tumors in irradiated mice. Female mice receiving 100 to 400 r were sterile after producing 1 litter (*33*). It may be assumed from this that the females of the present experiment were also sterile, yet histologic observations showed that spermatogenesis was taking place in most males irradiated at maturity. Yet no sex difference in adrenal-tumor incidence was noted. The males irradiated at birth were probably sterile, yet fewer adrenal tumors appeared in these males than in the males of other groups. The mice protected with cysteine and anoxia had as high an incidence of adrenal tumors as the other groups of mice, and the incidence in the other groups did not appear to be dependent on dose. Hornykiewytsch (*34*) found that pretreatment with cysteine prevented early irradiation change in the adrenals following 800 to 1000 r. It is difficult to interpret these results as a mere castration effect.

The induction of adrenal tumors by irradiation was not strain- or species-dependent. Koletsky (*35*) found adenomas, carcinomas, and neuroblastomas of the adrenals in rats surviving an LD70 dose of X irradiation. Upton, Furth, and Christenberry (*11*) reported an increase in adrenal adenomas following X irradiation or thermal neutron irradiation of RF mice. Similarly, polonium administration produced adenomas of the adrenal medulla (*36*).

The increase in lung tumors and hepatomas noted following chronic *gamma* irradiation (*24*) was not observed after the single doses given in the present experiments. Differences in life span, total dose, and strain make a comparison of results difficult. Upton *et al.* (*11*) found an increase in both lung tumors and hepatomas in RF females but not in males following doses up to 512 r or equivalent neutron radiation. No explanation can be given for the low incidence of hepatomas in the sham spleen-protected males.

The increase in mammary carcinomas but not in mammary sarcomas was in line with the findings of Furth *et al.* (*10*) and can conceivably be attributed to shortened life span. In all groups the sarcomas appeared later than the carcinomas.

Acute doses of X radiation were less leukemogenic to C3Hf mice than was chronic *gamma* irradiation (*24*). The proportion of lymphocytic neoplasms involving the thymus was about the same in both cases.

Although, in the present study, the number of neoplasms of reticular tissues was small, the results confirmed 1 observation of other investigators (*37,38*), namely, that radiation-induced lymphosarcomas were inhibited by spleen protection. The lower incidence of lymphocytic neoplasms in the 900 r spleen-shielded group as compared with that of the 400 r spleen-protected group cannot be attributed to the shorter survival time of the former animals, for lymphocytic leukemia occurred early.

The number of lymphosarcomas was the only notable difference between chemical protection and spleen protection, as far as carcinogenesis was concerned. Cysteine and anoxia did not protect completely against lymphosarcoma induction. The lymphocyte count of these animals was depressed to a greater degree than that of the spleen-protected animals. However, Gerebtzoff and Bacq (*39*) found that spleen, liver, and thymus recovered earlier when cysteinamine was administered prior to irradiation. Spleen protection may also have altered the granulocytic-leukemia incidence, but no difference was observed in the granulocyte count of the 900 r spleen-protected mice and the cysteine+anoxia protected mice.

Kaplan and coworkers (*40*) found that cortisone and testosterone decreased, and orchiectomy increased, radiation-induced thymic lymphosarcomas. They also found that ovariectomy had no effect, and estrogen administration increased the incidence in some strains. Upton and Furth (*41*) noted that cortisone altered the number of radiation-induced lymphocytic neoplasms but did not affect the incidence of granulocytic leukemias in RF mice. Orchiectomy increased the number of lymphosarcomas and decreased the granulocytic-leukemia incidence, while ovariectomy decreased the incidence of lymphosarcoma but did not influence granulocytic leukemia in irradiated RF mice (*42*).

In the present study, the difference in leukemia incidence probably could not be attributed to a difference in castration effect or adrenal response of the spleen-shielded animals.

Kidney carcinomas previously have been reported to be induced by radiation in both mice (*10*) and rats (*35*). Harderian-gland tumors were also elicited by irradiation but were not seen in the controls. In the present series some of the Harderian-gland tumors were found only upon examination of histologic sections. Because the Harderian gland of the controls was not examined routinely, some tumors of this type may have been missed. Similarly, in this study the absence of pituitary tumors, such as seen by Upton and Furth (*27*), can conceivably be attributed to the lack of routine examination of this organ.

Various miscellaneous carcinomas and sarcomas at sites such as the uterus, pylorus, cecum, and stomach may also be ascribed to irradiation, since similar neoplasms were not found in the controls. However, the number of these tumors was too small to permit any conclusions. From studies on rats protected by parabiosis or 4′-aminopropiophenone and irradiated, Brecher *et al.* (*43*) and Finerty (*44*) concluded that irradiation elicited tumor types not found in the controls.

Summary

Studies on survival of C3Hf mice following single doses of X radiation showed that:

1) Control females lived longer than control males, but no sex difference in survival time was noted in the mice irradiated at birth or in those irradiated while undergoing actual or sham spleen shielding. Male mice protected with anoxia and cysteine against 900 r lived longer than females similarly treated.

2) Mice irradiated at maturity lived longer than mice irradiated at birth.

3) Long-term survival time was not influenced by spleen protection but was dependent on dose.

4) Cysteine and anoxia protection produced a dose-reduction effect reflected by a life span longer than that seen in spleen-protected mice receiving equivalent radiation treatment.

5) A given dose of radiation was more carcinogenic to females than to males. The number of ovarian and adrenal tumors was consistently increased. Carcinogenicity was influenced by spleen protection only in that lymphosarcoma induction was decreased. Tumor types not seen in control animals were seen in irradiated mice.

6) Glomerulosclerosis was prevalent in mice receiving radiation doses that are ordinarily lethal, while pyelonephritis in the males was reduced by all irradiation treatments.

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Some Observations on Biological Effects of Deuterium, with Special Reference to Effects on Neoplastic Processes [1, 2]

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The profound biological consequences attending the deuteration of living organisms were noted very soon after the discovery of the heavy isotope of hydrogen. Urey (*1*) clearly recognized that the changes in rate of chemical reactions that result from the replacement of hydrogen by deuterium would have particular significance for biological systems. Lewis (*2*) first established that the extensive substitution of deuterium for hydrogen in higher plants and animals had dramatic consequences and that such substitution, carried to extremes, may be incompatible with survival. Since that time, a large number of miscellaneous observations relating to the biological effects of deuterium have appeared in the literature. These were the subject of an admirable and most useful review by Morowitz and Brown (*3*). Of particular significance to the present work are the early studies of Barbour and coworkers on the pharmacologic action of deuterium oxide in mammals. In addition to examining the physiologic effects attending the deuteration of mice, Barbour and coworkers investigated the effects of deuteration on the survival of mice inoculated with carcinoma and lymphosarcoma. All of these studies have been summarized by Barbour (*4*) in a review paper. Very little work since then has been specifically directed to the elucidation of the complex physiologic consequences of deuteration in living systems. This situation is in contrast to the very numerous investigations in which deuterium has been employed in very low concentration as a tracer; such tracer studies are of no further interest in this investigation.

Until very recently, the difficulty of separating deuterium has limited the amount available for the study of its biological effects. At the present time, because of requirements in the field of nuclear energy, extensive facilities are in existence for the production of heavy water (*5*). Since

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deuterium oxide is now available by the ton at a price of a few cents per gram, it becomes opportune to re-examine questions regarding the biological effects of deuterium and to contemplate experimental work on a scale that was impossible for early investigators.

The Deuterium Isotope Effect

The basis for the biological effects of deuterium lies in the isotope effect on the relative rates of reaction of hydrogen and deuterium. Deuterium differs from hydrogen to a very appreciable extent in both its thermodynamic and kinetic properties. In general, bonds to deuterium will be ruptured less readily than bonds to hydrogen, primarily, but not exclusively, because of differences in zero-point energy. Because of the greater stability of deuterium bonds, the rate of scission of such bonds will generally be slower than for corresponding hydrogen bonds. Thus, on the basis of calculations from absolute-rate theory, a -C-H bond may undergo cleavage about 7 times faster than a -C-D bond, >N-H 8.5 times faster than >N-D, and O-H 10.6 times faster than O-D. These values are maximal for the isotope effect at 25° C. The isotope effect decreases with increasing temperature, and the maximum isotope effect is, of course, not observed in all reactions. Significant isotope effects have been observed in the rates of: (1) acid- and base-catalyzed reactions; (2) various enzyme-catalyzed reactions; (3) oxidation reactions in deuterium oxide. A large number of investigations have confirmed the reality of the deuterium isotope effect, and this literature has recently been the subject of a comprehensive review (*6*).

The change in reaction rate occasioned by the replacement of deuterium for hydrogen is particularly important in biological systems. Experiments have shown the rates of various enzymatic reactions *in vitro* to be subject to the deuterium isotope effect (*7*). It is not surprising that even relatively minor changes in the rates of various enzymatic reactions should have far-reaching consequences in closely linked and highly complex biological systems. While it is unlikely that all enzymatic processes will be affected to an equal extent or even in the same direction by deuteration, it is reasonable to suppose that enzymes involved in hydrogen transport will be particularly influenced by deuteration. Moreover, any appreciable perturbation in the rates of even a small number of enzyme systems may result in serious difficulties for the organism. Similar kinetic effects may be expected to occur as the result of substitution of C^{13}, N^{15}, or O^{18} for the normal isotopes present in tissue. Although these effects *in vitro* are much smaller than with deuterium, nevertheless, isotopic substitutions for carbon, oxygen, or nitrogen may likewise be of very considerable interest in biological systems.

Although deuteration may on occasion cause an increase in the rate of a given single reaction, the over-all effect appears to be a depression of tissue metabolism. This depression in metabolism is a consequence of the fact that the deuterium isotope effect is in the direction of reduced reaction rates. (Apparent increases may in reality be the consequences

of depression of inhibitory processes.) Studies with tissue cultures (*8,9*) and mice (*10*) support the view that deuterium, especially at elevated concentrations, depresses biological activity. The data from these sources indicate that the toxicity of heavy water is not manifested until body fluids are 25 to 30 percent deuterated but that above this level toxicity increases rapidly with increasing deuterium concentration.

Earlier workers have shown that deuteration also inhibits neoplastic growth (*8,11*). Cultures of mouse carcinoma cells were unable to grow in 50 percent heavy water. The rate of tumor growth in mice drinking 40 percent deuterium oxide was one half as fast as that of controls, but the deuterated mice showed slower growth and earlier death. The premise of the present work is that the isotope effect may manifest itself to a different extent in normal and neoplastic tissues. A rapidly metabolizing tissue may be affected to a greater extent than is normal tissue by a reduction in metabolic activity. Deuteration may thus preferentially inhibit neoplastic growth.

Materials and Methods

Young adult CF-1 (Carworth Farms) female mice and 4- to 6-month-old female rats of the Sprague-Dawley strain were used in these studies. Deuterium was provided *ad libitum* in the drinking water. Rats were given 99.6 percent D_2O for 3 days and, thereafter, they were placed on a 50 percent deuterium oxide solution. In the experiments with hypophysectomized rats, which were used 14 days after operation, a solution of 20 percent sucrose in 50 percent deuterium oxide was supplied as drinking water, and the diet was supplemented with raw carrots. Rat urine was collected in metabolism cages and mouse urine was collected by manual expression from the bladder. Analyses were carried out on tissues from sacrificed animals and, in some experiments, on blood obtained by cardiac puncture under ether anesthesia. The methods employed in rat-tissue enzyme assays are listed elsewhere (*12,13*); these procedures were carried out on rats deprived of food for 24 hours.

Deuterium oxide was purified in a Barnstead conductivity-water apparatus by distillation of 99.6 percent material. The distillation from alkaline permanganate was carried out in 10 l. batches. The tritium content of the deuterium oxide was less than 0.01 μC per gm. A knowledge of the tritium content of deuterium oxide is important, since heavy water that has been in a nuclear reactor may contain tritium formed by neutron capture far in excess of the maximum permissible level of 0.2 μC of tritium per ml. of ordinary water.

The deuterium content of urine, serum, and various aqueous samples was determined spectrophotometrically in micro-cells constructed for use with a Beckman Model DK recording spectrophotometer. The cells were machined from ⅞-inch (gray) Teflon rod, provided with 2 filling holes (to take a 27-gauge needle), and fitted with ⅜-inch $\times$ 1 mm. quartz windows. The volume of the sample required was approximately 10 μl.; the optical path was 1 mm.

Concentrations of deuterium were estimated, by a modification of existing methods, from the difference in optical density between absorption peaks in the near infrared range at 1.44 and 1.66 μ (*14,15*). The cells were calibrated with known mixtures. Unlike the density and refractive-index methods for deuterium determination, tedious sample purification is unnecessary. The useful range of this infrared method is from 5 to 60 atom percent deuterium. In this region, replicate analyses of urine and of tissue distillate showed a sample standard deviation of 0.6 atom percent. Below 5 atom percent deuterium the concentrations were approximated by means of the transmission at 1.44 μ. All results are expressed as atom percent deuterium. It should be emphasized that in this paper, unless otherwise specified, the extent of deuteration of the animal refers to the deuterium content of the urine, serum, or tissue fluid.

Serum and clear urine samples were analyzed directly. Turbid urine samples were usually clarified in a microcentrifuge, and particularly dark urine was decolorized with charcoal and centrifuged prior to anlysis. The deuterium content of water distilled from 15 assorted urine and serum samples was determined and compared with the value obtained by direct analysis. The distillates showed a consistent average of 2 to 3 atom percent less deuterium. This difference is to be expected, since any factor affecting the optical density, such as turbidity or the presence of a solute, would affect the deuterium estimation.

Water distilled from various tissues was also analyzed for deuterium. The distillations were performed under vacuum ($<10^{-5}$ cm. Hg) in small h-shaped vessels. Distillation of small samples of water of known deuterium content (from 20 to 50%) indicated less than 1 atom percent dilution with H_2O under the conditions of manipulation. In combustion analyses, the tissues were finally dried in vacuum at 105° C. for 24 hours. The combustion procedure, a modification of that used by Smith, Trace, and Barbour (*16*) consisted of oxidation in an ordinary combustion furnace and collection of the water in a U-tube immersed in carbon dioxide snow.

Results

The course of deuteration.—The rate of deuteration of mice is indicated in text-figure 1, in which mean deuterium concentration in urine is plotted against number of days on heavy water. The deuteration quickly approached a steady state determined by the deuterium ingested in the drinking water and the hydrogen content of the food.

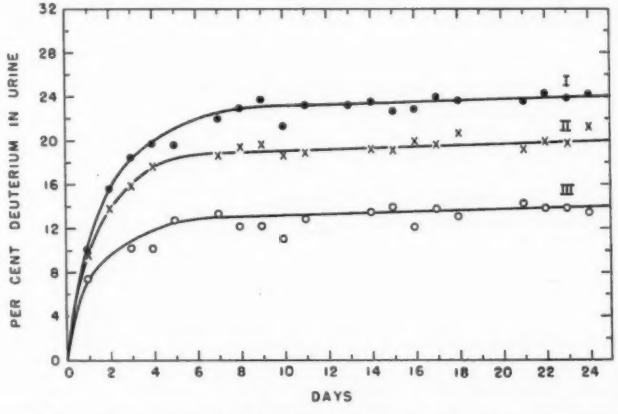
After intake of 30 and 25 percent deuterated (v/v) drinking water for 3 weeks, the deuterium level in urine was 79.0 percent of that in the ingested water; with 15 percent deuterated water, the urine level was 93.3 percent compared to ingested water. The lower the deuterium content of the drinking water, the more closely the deuterium content of the urine and water approached each other. These data undoubtedly reflect a lower intake of the more highly deuterated drinking water, a finding consistent with the observations of Barbour and Allen (*11*).

Tissue distribution of deuterium.—Analyses of water obtained by distillation at 25° C. of selected mouse tissues yielded the results shown in table 1. These tissues were obtained from mice at their equilibrium deuteration levels.

TABLE 1.—*Ratio of deuterium content of fluid from selected mouse tissues to deuterium content of urine at equilibrium*

Deuterium in urine (*atom per-cent*)	Liver		Spleen		Kidney		Brain	
	No. of mice	Ratio	No. of mice	Ratio	No. of mice	Ratio	No. of mice	Ratio
23–24	17	0. 933	16	0. 916	17	0. 918	10	0. 928
13–14	16	0. 867	14	0. 937	16	0. 929	10	0. 868

Table 2 shows the preliminary results of combustion experiments designed to indicate the extent to which deuterium is fixed in the tissues. The results are in general agreement with those obtained by Smith, Trace, and Barbour (*16*) and demonstrate that deuterium is indeed slowly fixed in stable positions in the tissues of animals subjected to deuteration. We attempted to determine the percentage of nonexchangeable deuterium in liver tissue. Homogenized livers were soaked for 6 hours at 25° C. in 10 volumes of water, soaked again for 24 hours in 10 volumes of water, lyophilized, and dried at 105° C. in vacuum. Combustion of this material indicated that only 20 percent of the deuterium had been exchanged. From work with isolated proteins (*17,18*) it might be anticipated that perhaps 80 percent of the deuterium would be in exchangeable positions. Further work is being done along these lines.



TEXT-FIGURE 1.—The course of body-fluid deuteration of mice with constant deuterium composition of the drinking water. Each point represents a mean of 5 to 22 determinations of urinary deuterium. Curve I, based on a total of 168 determinations, represents animals on 30 atom percent deuterium in the drinking water. Curves II and III, based on 301 and 158 determinations, respectively, represent mice on 25 and 15 atom percent deuterium in the drinking water.

TABLE 2.—*Deuterium content of dried tissues and of tissue fluids as a function of number of days mice received D_2O*

Animal	Days on 30 atom percent D	D_2O concentration in urine (*equil. value*)	D_2O concentration in terminal serum†	Water from distillation				Water from combustion			
				Liver	Kidney	Spleen	Brain	Liver	Kidney	Spleen	Brain
D4D54*	9	18. 6	21. 2	22. 5	21. 6	21. 2	22. 9	6. 7	6. 4	—	2. 9
D2C38*	26	25. 6	26. 5	25. 3	25. 6	24. 7	25. 3	8. 9	10. 1	11. 0	5. 1
D2C39*	26	24. 2	24. 1	21. 3	21. 3	22. 1	23. 0	8. 7	9. 4	9. 4	5. 1
D4A36	14	—	22. 4	21. 6	20. 9	20. 3	22. 2	5. 8	5. 1	6. 2	3. 1
D2C40	26	—	23. 7	22. 2	21. 5	21. 9	21. 7	7. 6	7. 4	8. 0	3. 6

*These tissues were dried for 24 hours at 95 to 105° C. in air.
†Atom percent deuterium in serum at time of sacrifice.

General consequences of deuteration.—The findings of Barbour and Trace (*10*) on the toxicity of heavy water in mice have been confirmed and were extended to rats. As the concentration of heavy water in the body fluids increased beyond 20 atom percent, signs of hyperirritability were observed first. As the concentration of heavy water approached 30 atom percent, symptoms of weakness, neuromuscular hyperexcitability, and pilomotor stimulation became general. Bradycardia, stupor, and death supervened as the level of deuteration reached 30 to 35 atom percent. Body weight of mice was essentially unaffected by levels of deuteration maintained by 30 atom percent deuterium in the drinking water. Rats receiving 50 atom percent deuterated water showed a slight progressive weight loss that became precipitous shortly before death.

It should be pointed out that we have concerned ourselves primarily with subacute-toxicity studies. We have no new information on the acute toxicity induced by rapidly bringing the animal to levels of 40 atom percent as quickly as possible. Under such conditions the animals become very sick, their body temperature drops sharply, all physiologic functions seem greatly depressed, and death occurs within a short time (*4*). We do not know whether these 2 modes of toxic response are identical or whether there exists a specific acute-toxicity mechanism.

Samples of blood or plasma from rats fed deuterium contained progressively increasing concentrations of inorganic phosphorus, nonprotein nitrogen, urea, and lactic acid. Blood glucose and plasma protein levels were decreased. The alkali reserve of the plasma was not greatly altered. In moribund rats, with plasma deuterium levels of 30 to 34 atom percent, both nonprotein nitrogen and urea were more than doubled while glucose was reduced by half and protein by one fifth.

We know that the dissociation of weak electrolytes in deuterium oxide differs markedly from that in ordinary water; therefore, we considered the possibility that the toxic symptoms in deuterated organisms might be related to disturbances in the carbon dioxide-bicarbonate equilibrium. To examine this point, 4 mice that had been deuterated to the extent of 20 atom percent and 5 control mice were placed in an 80 percent oxygen-20 percent carbon dioxide atmosphere. Immediately all the animals became tachypneic. However, in contrast to the control mice, the deuterated mice showed, in sequence: (1) marked neuromuscular hyperexcitability within 1 to 3 minutes; (2) narcosis beginning between 3 and 5 minutes; (3) gradual change of respiration from marked tachypnea to hypopnea; (4) marked bradycardia after 5 to 10 minutes; (5) deepening narcosis. Removal of the animals to room air resulted in the loss of the above symptoms in reverse of their order of occurrence. We conclude that deuteration does in fact alter the response of animals to high concentrations of carbon dioxide, but how this relates to the toxicity of deuterium remains unknown.

Rats killed by elevated deuterium levels showed an increase in adrenal size from the normal average of 0.0239 percent of total body weight ($\sigma = 0.0021$) to values approaching 0.05 percent. This adrenal hyper-

trophy suggests that heavy water was acting as a nonspecific stress. Hypophysectomized rats were much more susceptible to the toxic effects of heavy water, since they died when the plasma contained 16 to 24 atom percent deuterium. The increase in urea and nonprotein nitrogen and decrease in glucose and plasma protein were the same as in unoperated deuterated rats. In the hypophysectomized rats these changes developed within 8 days, whereas in normal rats consuming similar amounts of heavy water 25 days were required.

Effects on liver and kidney.—Liver enlargement was noted in both mice and rats deuterated to the limits of toleration. Some rats showed jaundice. The size of the liver, expressed as a percentage of total body weight, increased from a normal average of 2.41 ($\sigma = 0.12$) to an average of 3.73 ($\sigma = 0.47$) in 3 rats sacrificed when their plasma deuterium levels were 30 to 32 atom percent (table 3). In rats less heavily deuterated, the increase in liver size was less marked. The increase in liver size did not become significant until the deuterium levels of the plasma exceeded 15 atom percent. There was a very high positive correlation ($r = 0.90$) between liver weight and adrenal weight in the deuterated rats. We do not have a definite explanation for the fluctuations in the time course of deuteration, but they are probably referable to variations in the ratio of food to water intake in the different groups of rats used in these experiments. Livers of hypophysectomized rats were not measured.

The dry weight of the liver, as percentage of fresh weight (measured on deuterated rats fasted 20 to 24 hours before sacrifice), did not vary significantly from control values. Thus, the increase in total weight of the liver cannot be attributed solely to increased water content. Similarly, the concentration of total nitrogen per unit dry weight was not significantly altered. Measurements of the deoxyribonucleic acid phosphorus (DNA-P) of the livers of deuterated rats showed that the increase in liver mass was due largely to an increase in number rather than size of cells, since the DNA-P concentration was virtually unchanged.

Liver-function studies on less heavily deuterated mice indicated little abnormality. Bromsulfalein (bromsulfonphthalein, BSP) excretion was studied according to a method for following serial blood BSP levels in mice (*19*). Mice were deuterated by feeding 25 percent D_2O in the drinking water. Blood BSP retention was measured at weekly intervals. The mean BSP-retention curve so obtained was slightly higher for the deuterated mice than for control mice. This difference is of doubtful significance. Glutamic-oxalacetic transaminase (GOT) activity of serum has been employed as an index of liver-cell injury (*20*). The serum GOT values obtained with mice deuterated to the extent of 13 to 14 and 23 to 24 atom percent clearly fell within the range for normal mice and confirm the absence of hepatocellular damage at these levels of deuteration. Similarly, alkaline-phosphatase (*21*) and cholinesterase (*22*) values of serum were essentially the same in normal mice and in mice deuterated to 20 atom percent. Thymol-turbidity (*23*) and cephalin-cholesterol flocculation (*24*) tests failed to distinguish deuterated from normal mice.

TABLE 3.—*Effect of deuterium oxide on wet weight of rat liver and adrenals and on dry weight and nitrogen content of rat liver**

Days on 50 percent D_2O	Plasma D (*atom percent*)	No. rats	Total body weight (% *initial wt.*)	Liver wet weight (% *total body wt.*)	Liver dry weight (% *wet wt.*)	Liver N (*mg./gm. wet wt.*)	Adrenal wet weight (% *total body wt.*)
0	—	14	—	2. 41±0. 12†	28. 9±1. 45†	34. 1±2. 85†	0. 0239±0. 0021†
8	17. 4	4	**101. 6** (100. 0–102. 8)	**2. 48** (2. 43–2. 58)	**29. 1** (28. 5–31. 2)	**30. 0** (28. 8–31. 2)	**0. 028** (0. 026–0. 030)
9	26. 2	3	**95. 8** (95. 6–96. 0)	—	**29. 0** (28. 2–29. 8)	**31. 3** (30. 0–32. 6)	**0. 034** (0. 031–0. 036)
14	27. 0	3	**96. 2** (93. 6–101. 2)	**2. 80** (2. 63–2. 96)	**26. 3** (25. 9–26. 5)	**29. 2** (28. 4–30. 0)	**0. 032** (0. 030–0. 035)
17	19. 0	4	**87. 8** (85. 2–95. 6)	**2. 89** (2. 74–3. 20)	**27. 0** (25. 9–28. 4)	**31. 7** (28. 8–32. 6)	**0. 036** (0. 030–0. 042)
18	25. 3	4	**87. 5** (85. 0–91. 9)	**2. 96** (2. 59–3. 27)	**27. 5** (26. 0–28. 6)	**31. 7** (30. 0–33. 2)	**0. 033** (0. 025–0. 041)
23	32. 2	3	**90. 1** (87. 9–91. 9)	**3. 73** (3. 39–4. 27)	**27. 2** (26. 0–28. 6)	**33. 6** (30. 8–37. 0)	**0. 046** (0. 043–0. 053)
24	28. 3	3	**92. 8** (91. 7–94. 2)	**2. 94** (2. 79–3. 22)	**30. 0** (29. 2–31. 2)	**33. 5** (33. 0–33. 8)	**0. 035** (0. 032–0. 036)
26	29. 1	3	**91. 1** (85. 0–95. 2)	**3. 29** (2. 83–3. 56)	**27. 9** (27. 6–28. 4)	**34. 5** (32. 4–36. 4)	**0. 036** (0. 029–0. 042)
32	27. 4	3	**92. 7** (87. 6–97. 8)	**3. 30** (3. 13–3. 42)	**27. 3** (27. 2–27. 6)	—	—

*Figures in bold face = average values; figures in parentheses = range of values.
†Mean ± standard deviation.

The kidneys of rats succumbing to deuterium poisoning were grossly enlarged and pale. Histologic examination showed marked destruction of the tubules but revealed no essential changes in the glomeruli. Microscopic examination of the urine was negative. These findings are consistent with the observations of Dybing, Guldberg, and Hansen (*25*), who found mild pathologic changes in mouse liver, kidney, and spleen as the level of deuteration approached 30 atom percent.

Thus it appears that no serious liver and kidney disturbances are observed in animals maintained for periods up to several weeks at deuterium levels below 25 atom percent.

Effect of deuteration on enzyme assays.—The results of assays on various rat-liver enzymes are listed in table 4. The concentrations of arginase and uricase were increased as much as 20 percent, depending on the level of deuteration. Slight decreases in concentration were noted for succinic dehydrogenase, cytochrome oxidase, and adenosinetriphosphatase. More striking decreases (20–35%) were observed for esterase,

TABLE 4.—*Effect of administration of D_2O on relative activities of certain enzymes of rat liver*

Days on 50 percent D	Plasma D_2O (*atom percent*)	Enzyme activity as percentage of control (based on dry weight)						
		Catalase	Cytochrome oxidase	Succinic dehydrogenase	DPN-cytochrome *c* reductase	Esterase	Uricase	Arginase
0	—	100	100	100	100	100	100	100
8	17. 4	100	—	105	100	81	106	—
9	26. 2	75	89	89	77	—	105	—
14	27. 0	66	90	103	67	—	113	—
17	19. 0	70	90	83	75	84	109	118
18	25. 3	74	—	—	71	76	131	—
22–24	32. 2	67	—	—	66	—	—	—

DPN-cytochrome *c* reductase, and catalase. However, despite the decreases in concentration, the total activities of these last enzymes (per unit body weight) were diminished less than 10 percent because of the increase in liver size previously described.

In the rat kidney, a slight decrease in succinic-dehydrogenase activity was noted. No changes were observed in the concentration of DPN-cytochrome *c* reductase, cytochrome oxidase, or catalase in either kidney or brain. We have not measured cholinesterase in the brain.

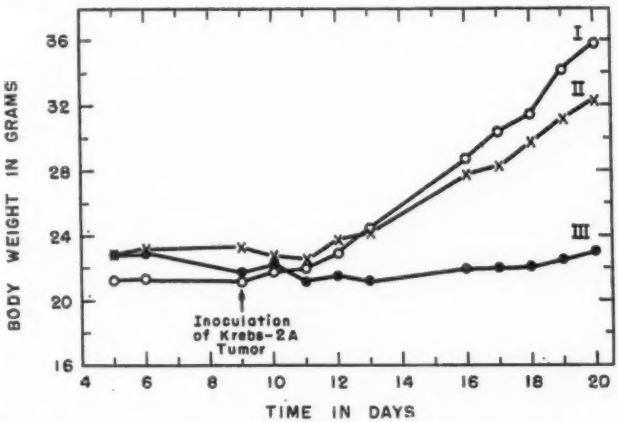
The capacity of mitochondria of liver and kidney to esterify phosphate was studied in rats deuterated to 30 atom percent. Although the amounts of oxygen consumed and of phosphate esterified were decreased 10 to 25 percent on a nitrogen basis, in neither tissue was the ratio of phosphorus esterified to oxygen consumed significantly altered for any of the 3 substrates employed; namely, succinate, fumarate, and α-ketoglutarate.

The possibility exists, of course, that some of these enzymes may have been inactivated in the animal but restored to activity by exchange with

hydrogen under the conditions of the assay methods employed. However, when we assayed several of these enzymes from normal rat liver in the presence of 30 percent D_2O, we observed negligible alterations in activity.

Although no striking consequences have so far been observed in the enzyme systems studied, the possibility exists that a more extensive investigation along these lines may reveal an enzymatic basis for the toxicity of deuterium.

Ascites-tumor studies in mice.—Early experiments by Barbour and Allen (*11*) indicated that lymphosarcoma and mammary carcinoma of mice could be inhibited by deuterium, although at the deuteration level employed the animals also suffered from deuterium poisoning. We have conducted experiments with tumors transplanted into animals deuterated to the extent of 13 to 14 and 23 to 24 atom percent. In a preliminary experiment in which Krebs-2 ascites tumor was implanted subcutaneously (in an effort to obtain a solid tumor), a number of mice developed ascites. Using body-weight changes (text-fig. 2) to estimate the growth of the ascites tumors (*26*), we found that tumor growth was inhibited by increasing deuteration.



TEXT-FIGURE 2.—Mean body weights of mice bearing Krebs-2 ascites tumors.
Curve I. Control mice fed H_2O.
Curve II. Mice maintained at 13 to 14 atom percent deuteration throughout experiment.
Curve III. Mice maintained at 23 to 24 atom percent deuteration throughout experiment.

More definitive data were acquired from an experiment in which an intraperitoneal inoculum of 7×10^5 cells was used in animals maintained at the 13 to 14 and 23 to 24 atom percent deuterium levels. These animals had been deuterated to the maintenance level during the previous week. The growth characteristics of the ascites tumors were analyzed quantitatively by the methods developed by Patt *et al.* (*27*) and are given

TABLE 5.—*Growth characteristics of Krebs-2 ascites tumor in deuterated and nonaeuterated CF-1 mice**

Day	Deuterium in body fluids (*atom percent*)	No. of mice	Mean cell concentration (*cells/ml.* × 10^6)	Mean total ascites-fluid volume (*ml.*)	Mean total tumor cells (× 10^6)	Mean tumor-cell volume† (*ml.*)
4	0	1	68. 84	2. 17	149. 4	0. 51
	13–14	3	70. 94	2. 86	202. 9	0. 68
	23–24	3	59. 29	3. 10	183. 8	0. 72
7	0	3	91. 81	6. 86	629. 8	2. 07
	13–14	3	85. 90	5. 41	464. 7	1. 97
	23–24	2	92. 96	3. 85	357. 9	1. 55
9	0	3	86. 43	14. 33	1238. 6	7. 75
	13–14	2	76. 65	9. 44	723. 6	4. 38
	23–24	3	66. 17	5. 14	340. 1	2. 81

*Inoculated intraperitoneally with 7×10^5 tumor cells.

†Mean tumor-cell volume is derived by multiplying the total ascitic-fluid volume by the percent packed-cell volume (with a correction for the dilution by the dye used in the procedure for volume determination).

in table 5. The results for mean total ascitic-fluid volume, mean total tumor-cell population, and mean tumor-cell volume clearly indicate that ascites-tumor growth is inhibited by deuteration.

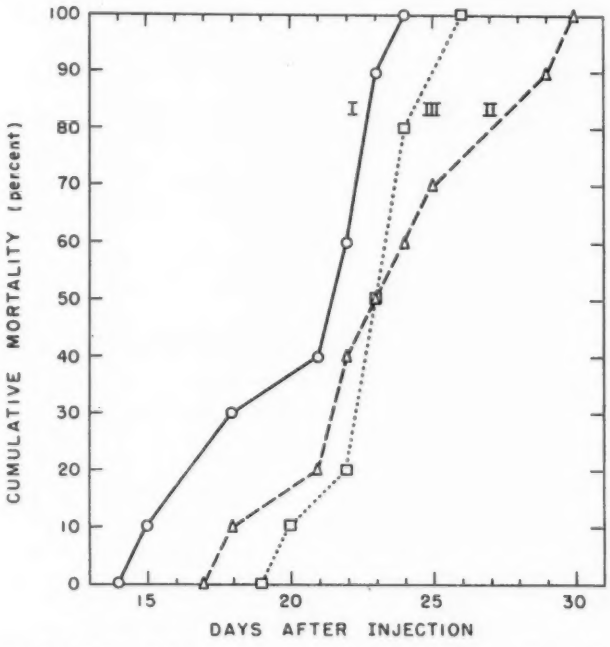
The effect of deuterium on ascites-tumor growth can be postulated to be a result of either inhibition of cell division or destruction of the individual cells. Some insight into this problem is afforded by an examination of the data on glutamic-oxalacetic transaminase (GOT) in table 6. It can be seen that in the animals with the subcutaneous solid tumors the serum GOT data are within the range for normal mice (20 to 120 units in our experience). The ascites-bearing animals have a somewhat increased mean serum value in the nondeuterated group and increasingly elevated serum values with increasing levels of deuteration. The GOT levels in the ascites plasma increased from a mean of 120 units in the nondeuterated mice to values in the deuterated animals that were substantially higher than those found in the corresponding blood sera. If GOT can indeed be used as a measure of cell destruction, as it has been used in the study of clinical and experimental heart and liver-cell injury,

TABLE 6.—*Glutamic-oxalacetic transaminase values of mouse sera and ascites plasma*

Deuterium in body fluids (*atom percent*)	Glutamic-oxalacetic transaminase values				
	Ascites-phase tumors			Solid-phase tumors	
	Mean serum values	No. of mice	Mean ascites-plasma values	No. of mice	Mean serum values
23–24	282	5	461	7	86
13–14	202	8	238	7	101
0	142	9	120	4	90

these data indicate that deuterium in the ascitic plasma at appropriate levels leads to tumor-cell destruction. The enzyme, presumably released into the ascitic plasma with cell injury, is carried into the general circulation, with consequent elevation, to a lesser degree, of the serum GOT values.

Effect of deuteration on mouse leukemia.—Work by Simmons and Jacobson (*28*) on the leukemia induced in DBA/2 mice by the intraperitoneal injection of specific numbers of splenic cells from mice dying of P-1534 lymphatic leukemia indicates that a family of cumulative-mortality curves with parallel slopes can be drawn. The positions of these curves will be dependent on the number of leukemia cells originally injected. Accordingly, 3 groups of DBA/2 mice were injected intraperitoneally with 10^5 splenic cells. Group 1 consisted of 20 control mice given ordinary water at all times; group 2, 10 mice deuterated to 24 to 26 atom percent before injection and maintained at this level until death; group 3, 10 mice fed ordinary water until injected and thereafter gradually deuterated. In text-figure 3, the data are plotted as percent cumulative mortality *versus* day of death.



TEXT-FIGURE 3.—Effect of deuteration on mortality of mice from P-1534 leukemia.
Curve I. Control mice fed H_2O.
Curve II. Mice maintained at 24 to 26 atom percent deuteration throughout experiment.
Curve III. Mice given D_2O immediately after inoculation, brought up to 24 to 26 atom percent deuteration.

Half of the control mice (group 1) were dead after 20.5 days, whereas the length of time for comparable mortality in groups 2 and 3 was 23 days. In text-figure 3 it can be seen that there is a slight but consistent prolongation of life in the leukemic mice receiving deuterium. The *P* value of the difference in average survival times is 0.03. Deuteration appears to displace the mortality curve to the position normally expected after the injection of less than half the number of leukemic cells actually used on this occasion (*28*).

Effect of deuteration on radiation sensitivity.—One of the consequences that may be expected to follow extensive deuteration of a living organism is a change in radiation sensitivity. In the case of γ-radiation (*29*) and nuclear-reactor radiations (*30*), it has been shown that the free radicals formed from D_2O react more readily with other substances than do those formed from H_2O. The extent of chemical reaction will therefore be different in the 2 media. Furthermore, the cross-section for neutron capture by deuterium is well known to be very much less than that of ordinary hydrogen, and it has been estimated (*31*) that replacement of one fourth of the body hydrogen in the mouse by deuterium will render the animal 20 percent less susceptible to slow neutrons. It was therefore of some interest to examine the effects of different combinations of radiation and deuteration on the survival of X-irradiated mice.

Two hundred and forty CF-1 mice, 12 to 14 weeks old, were randomly divided into 24 groups for deuteration and irradiation. The animals were brought to the desired body level of deuterium before irradiation by supplying drinking water of varied deuterium content. All irradiations were done on the same day with a GE Maxitron X-ray unit operating at 250 kvp at 30 ma. with 0.25 mm. Cu and 1 mm. Al filtration of the beam. The radiation doses, in rads, were 550, 600, 650, 700, and 750. Body-water deuterium levels were 0, 8 to 10, 15 to 18, and 25 to 28 atom percent from the time of irradiation until the termination of the experiment. This experiment was repeated with doses of 700, 750, and 800 rads. The deuterium levels and the radiation factors were the same as in the first experiment. Each group consisted of 10 CF-1 mice of the same age and weight distribution, as described above.

The data from these 2 experiments (table 7) show that, for the most part, there was greater survival in animals that were deuterated to 8 to 10 and 15 to 18 atom percent. On the other hand, fewer animals survived in the groups deuterated to 25 to 28 atom percent than in those with lower deuterium levels. The results are, perhaps, to be expected, since this is the region in which the toxic effects of heavy water are rapidly multiplied. The variations in LD50, in spite of the limited number of animals employed, are of interest in view of the increased survival at intermediate levels of deuteration.

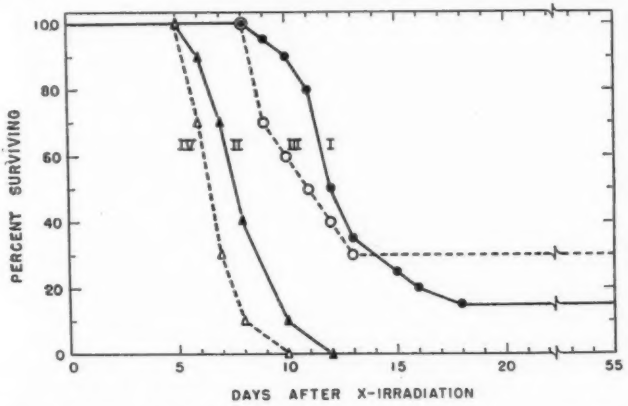
Another experiment was designed in an attempt to establish a schedule of deuteration that would give maximum protection against X irradiation. Four groups of animals received 750 rads total-body irradiation: group 1, 20 mice given ordinary water at all times; group 2, 10 mice maintained

TABLE 7.—*Effect of deuterium on survival after total-body X irradiation*

Expt.	Radiation dose (*rads*)	Percent survival (30 days) at following atom percent deuterium levels:			
		0	8–10	15–18	25–28
1	550	90	100	100	100
	600	100	90	70	50
	650	60	90	90	60
	700	40	70	70	60
	750	10	40	50	40
2	700	20	50	60	0
	750	20	20	40	0
	800	0	0	20	0
LD50* (*rads*)		669	713	730	651

*Calculated according to Bliss (*52*).

at a body-water deuterium content of 23 to 26.6 atom percent throughout the experiment, group 3, 10 mice brought to a body-water deuterium content of 23 to 26.6 atom percent before irradiation, maintained at this level until 4 days after irradiation, and then given ordinary water; group 4, 10 mice brought to a body-water deuterium content of 23 to 26.6 atom percent before irradiation, after which they were given ordinary water. The results of this experiment, illustrated in text-figure 4, indicated that only those animals that were deuterated before irradiation and for 4 days thereafter survived as well as the nondeuterated, control mice.



TEXT-FIGURE 4.—Effect of deuteration on survival of mice exposed to 750 rads total-body X irradiation.

Curve I. Control mice fed H_2O.

Curve II. Mice maintained at 23 to 26.6 atom percent deuteration from time of irradiation until death.

Curve III. Mice maintained on deuterium oxide until 4 days after irradiation, then given H_2O.

Curve IV. Mice transferred from D_2O to H_2O immediately after irradiation.

Discussion

In the course of the present work we have verified and extended many of the observations of Barbour concerning the effects of deuterium on the mammalian organism. We consider such verification desirable and important in view of the considerable confusion that exists in the literature dealing with the biological effects of deuterium. It has been shown that deuterium is easily absorbed, is toxic at elevated concentrations, and is slowly fixed in stable, nonexchangeable positions of compounds present in tissues. In short-term experiments, mice and rats may be deuterated to the 25 atom percent level before toxic symptoms appear. In the 30 to 35 atom percent region the animals die; this region of concentration is reached when the animals are fed for 4 to 6 weeks on water containing 50 atom percent deuterium. On the basis of our studies there would appear to be a fairly abrupt transition from the asymptomatic state, at 20 to 25 atom percent, to the toxic effects manifested above 30 atom percent. In a large organism, this 5 percent difference in deuterium content may actually represent several kilograms of deuterium and may constitute a sizable margin of safety.

Except for a few early experiments by Barbour (*4*), little has been done on the survival of animals maintained for long periods of time at and below concentrations of 25 atom percent deuterium. We have initiated studies concerned with the chronic effects of deuteration so that a clearer assessment of the toxicity of deuterium can be made.

At the present time we are unable to state the mechanism by which deuterium exerts its deleterious effect. Although a number of constituents of blood and plasma, such as glucose, protein, and nonprotein nitrogen, vary in a manner suggestive of liver damage, there is no significant alteration in liver function as determined by a variety of tests, nor is there any evidence of an impairment of the ability of liver mitochondria to esterify inorganic phosphate. Of a number of enzymes assayed in liver homogenates, several were found to be diminished in concentration, but in view of the fact that the total mass of the liver had increased, the diminutions are of questionable significance. It is possible that the increases in uricase and arginase represent adaptation to the acceleration of catabolic processes, manifested by the accumulation of nitrogenous constituents of blood. Regenerating rat liver, where cellular proliferation is extensive, presents a similar picture.

In animals that were killed by deuteration we have observed extensive lesions in the kidneys, although at lower levels of deuteration there appears to be no significant impairment of kidney metabolism. Despite the high levels of plasma nonprotein nitrogen, we have no specific evidence of impairment of renal function. Disturbances of the central nervous system, primarily hyperexcitability, become manifest at levels between 20 and 25 atom percent. Although the role of the central nervous system in the death of the animal is uncertain, there may be a relationship between terminal narcosis and lowered blood glucose.

The relationship between deuteration and radiation sensitivity is ex-

ceedingly complex. In certain experiments deuterium effected a slight prolongation of the lives of irradiated mice. Interest in this aspect of the subject derives from the possibility of modifying the effect of X irradiation by deuteration. From a consideration of fundamental principles it is reasonable to anticipate that deuteration may alter sensitivity to other varieties of radiation as well. Numerous possibilities exist also for combining deuteration with administration of compounds of pharmacologic interest.

At levels below the toxic limit, deuteration has been observed to retard neoplastic growths in mice. Growth of the Krebs-2 ascites tumor was retarded at both the 13 to 14 and 23 to 24 atom percent levels of deuteration. Determinations of glutamic-oxalacetic transaminase in blood serum and ascitic plasma suggested that these levels of deuterium in the animal led to tumor-cell destruction. In an experiment in which lymphatic leukemia was introduced into mice, a slight but significant increase in survival was noted in the deuterated animals. It would appear from these results that rapidly metabolizing tissues are more susceptible to the inhibitory action of deuterium than tissues metabolizing more slowly.

Summary

1) A simple infrared spectrophotometric method has been developed for the analysis of deuterium in biological media.

2) Satisfactory schedules for the achievement of predetermined levels of deuteration have been worked out. The extent of fixation of deuterium in various tissues has been explored.

3) Symptoms of deuterium intoxication in rats and mice include weakness, neuromuscular hyperexcitability, bradycardia, and, eventually, stupor and death; these symptoms occur at body deuteration levels of 30 to 35 atom percent.

4) The blood of deuterated rats showed elevated inorganic phosphorus, nonprotein nitrogen, urea, and lactic acid and decreased glucose and plasma protein. Hypophysectomized rats showed the same blood changes as did unoperated, deuterated rats, but the changes occurred much earlier.

5) Adrenal hypertrophy in deuterated rats suggested that heavy water acted as a nonspecific stress. Liver enlargement was noted in both mice and rats that had been deuterated, but the deoxyribonucleic acid-phosphorus content of the liver indicated that this increase in liver mass was largely the result of an increase in number rather than size of cells.

6) Liver-function studies, including BSP and serum GOT, failed to demonstrate any consistent abnormality in deuterated mice.

7) Decreases in concentration of esterase, DPN-cytochrome *c* reductase, and catalase and increases in arginase and uricase were observed in liver homogenates. No significant changes in enzyme concentrations were noted in the kidney or brain of rats.

8) Deuteration of host mice resulted in reduced growth rates of injected Krebs-2 ascites tumors and of inoculated P-1534 lymphatic leukemia.

9) At certain levels of deuteration, the sensitivity of mice to X irradiation was decreased slightly.

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Effect of Prolonged Administration of Spermicidal Contraceptives on Rats Kept on Low-Protein or on Full Diet[1,2]

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In a search for the factors that might be involved in the development of cervical cancer in women, attention was focused on the possible effect of spermicidal contraceptives. Since compounds that are thought to kill one kind of cell might be endowed with properties that modify the normal growth pattern of other cells, they might increase or provoke the development of tumors.

In view of the fact that dietary constituents influence the neoplastic properties of certain carcinogens, the investigation was carried out with rats kept on a full diet as well as with rats on a semisynthetic diet of low protein content.

Materials and Methods

Female rats of the Wistar strain, weighing 60 to 80 gm. at the beginning of the experiment, were used, and in all but 16 rats the experiment was started as soon as the vagina was opened. Ten rats in the treated group and 6 in the control group were adult females of about 150 gm. in body weight, each of which had delivered 1 litter. Because no difference was noted in the results obtained with virgin or nonvirgin animals, the results have been combined. Five or 10 rats were housed together and food and water were supplied *ad libitum*.

The full diet consisted of Purina dog chow. The composition of the low-protein diet is given in table 1.

The rats were allowed to live 14 to 18 months after the beginning of the experiment; the findings are reported on animals between the ages of 16 and 20 months. Complete *post-mortem* examinations were carried out on all animals that died during the experiment or were sacrificed at the end. The hearts, livers, spleens, kidneys, and adrenals of the sacrificed rats were weighed. Representative pieces of all internal organs (with the exception of bone and bone marrow) were fixed in 4 percent formaldehyde. The sections were stained with hematoxylin and eosin; other

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TABLE 1.—*Composition of the diet*

Basic diet	Percent	Vitamins per 100 gm. of diet	Mg.
Vitamin-free casein	7	Thiamin hydrochloride	1.0
Sugar	6.5	Riboflavine	0.8
Cornstarch	56	Niacin	4.0
Cellulose	12	*p*-Aminobenzoic acid	10.0
Lard	6.5	Inositol	1.5
Vegetable oil	8	Choline citrate	10.0
Salt mixture (U. S. P. XIV)	3	Pyridoxine hydrochloride	1.0
Cod-liver oil	1	Calcium pantothenate	2.0
		α-Tocopherol	2.0

stains were done when indicated. Eleven animals found dead in a state rendering autopsy unprofitable were not included.

The contraceptives, chosen because they are the most widely used in this part of the country, are designated here as A and B. The active ingredients of these preparations, as given by the manufacturer, are as follows:

Contraceptive cream A:
Boric acid, 2%
Oxyquinoline benzoate, 0.02%
Phenylmercuric acetate, 0.02%
in an emulsion of stearic acid, cetyl alcohol, glycerin, perfume, and de-ionized water, *p*H 4.4.

Contraceptive jelly B:
Ricinolic acid, 0.7%
p-Diisobutylphenoxypolyethoxyethanol, 1.0%
Boric acid, 3.0%
*p*H 4.5.

Up to the present time, the investigation comprises 2 experimental and 2 control series.

In the first set of experiments, 35 young rats received daily vaginal instillations of contraceptive A or B. Fifteen rats were kept on a full diet, the others on a low-protein diet. The contraceptive substance was administered by placing a small quantity on a cotton-wool swab, which was put into the vagina, moved once around the cervix, and removed. After 1 or 2 administrations the rat became accustomed to the treatment and no resistance was encountered.

In the second set of experiments 80 rats were kept on the low-protein diet, 30 receiving contraceptive A or B by vaginal instillation as in the previous experiment, 50 receiving the contraceptive substance orally by pressing about 1½ to 3 $mm.^3$ of the substance directly into the mouth from the contraceptive tube.

The controls used were 20 female rats kept on a full diet of rat cubes for 14 to 16 months and 40 rats fed the low-protein diet from the age of 2 to 3 months for 14 to 16 months.

TABLE 2.—*Body and organ weights of rats kept on full or on low-protein diet with or without contraceptive treatment*

Expt.	Diet	Contra-ceptive	No. of rats	Body weight (*gm.*)		Liver (*gm./100 gm.*)		Kidney (*gm./100 gm.*)		Spleen (*gm./100 gm.*)		Heart (*gm./100 gm.*)		Adrenal (*gm./100 gm.*)	
				Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
First	Full	A or B*	14	182 360	247	3. 1 4. 5	3. 85	0. 91 1. 10	0. 98	0. 20 0. 65	0. 31				
	Low-protein	A or B	15	120 355	249	4. 4 8. 1	5. 70	0. 78 1. 11	0. 84	0. 21 0. 48	0. 30				
Second	Full	None	16	243 440	326	3. 4 3. 7	3. 08	0. 49 0. 73	0. 65	0. 21 0. 37	0. 29	0. 23 0. 42	0. 32	10. 0 32. 0	20. 4
	Low-protein	None	31	153 435	274	4. 4 8. 9	5. 74	0. 69 1. 25	0. 82	0. 15 1. 25	0. 36	0. 37 0. 59	0. 47	12. 7 54. 2	25. 2
	Low-protein	A	40	160 434	280	3. 7 12. 6	6. 2	0. 63 1. 34	0. 84†	0. 16 0. 8	0. 32	0. 29 0. 70	0. 43	11. 3 33. 5	21. 5
	Low-protein	B	24	160 394	279	3. 3 8. 9	5. 8	0. 45 1. 07	0. 83	0. 20 0. 45	0. 31	0. 27 0. 55	0. 39	11. 2 27. 8	18. 4

*The groups of rats in the first experiment on the same diet are pooled because of the small number in each group.
†The weights of 3 kidneys enlarged by tumors are excluded.

Results

Table 2 shows the body weights and the organ weights as a percentage of body weight of the rats killed at the end of the experiment. The weight of the body, given in the table, is calculated by subtracting the tumor weight from the total body weight. In the first set of experiments there was no difference in the body weights of contraceptive-treated rats on a full or a low-protein diet. The body weights in both of these groups, however, were significantly lower than those of the rats in the second experiment, which were kept on a full diet without any treatment. They did not differ from the weights of untreated rats kept on low-protein diet in the second experiment. The body weight of the latter group was on the average slightly lower ($P=0.05$–0.02) than that of untreated rats on the full diet. In the second experiment the average body weight of rats on the low-protein diet was the same, with or without contraceptive treatment.

The livers of the rats kept on the low-protein diet were about twice as big as those of the rats on the full diet, whether or not the rats were treated with a contraceptive ($P<0.01$). On gross and microscopic examination the livers of rats on the low-protein diet in all cases showed fatty changes and in many instances cirrhosis. Deposition of ceroid was very marked in the cirrhotic livers. In many cases cholangiofibrosis was present and showed nodular, tumor-like arrangements in 2 untreated rats on the low-protein diet and in 17 rats treated with contraceptives. Of the other organs, the weight of spleens and adrenals did not differ in any of the groups; the hearts and kidneys were heavier in the groups fed the low-protein diet. The difference was significant when calculated as a percentage of body weight but was not significant when the calculation was based on the actual weight of these organs.

The occurrence of tumors is summarized in tables 3 and 4. The number of tumors occurring in an animal are given in such a manner that any type of tumor is registered as 1, even if several tumors of the same type were

TABLE 3.—*Occurrence of tumors in rats treated with contraceptives and kept on full or low-protein diet*

Diet	Contraceptive	Mode of application	No. rats	No. rats with tumor	Total no. tumors	Liver tumors	Breast tumors	Tumors of other organs
Full	None		16	1	1	—	1	—
Low-protein	None		39	16	17	2	13	2
Full	A	Vaginal	5	1	1	—	—	1
Full	B	Vaginal	9	2	3	—	1	2
Low-protein	A	Vaginal	10	8	18	2	3	13
Low-protein	B	Vaginal	9	8	15	1	6	8
Low-protein	A	Vaginal	19	13	26	2	9	15
Low-protein	A	Oral	29	23	38	4	17	17
Low-protein	B	Vaginal	10	9	19	3	8	8
Low-protein	B	Oral	18	12	22	5	9	8

TABLE 4.—*Localization of tumors in rats treated with contraceptives and kept on full diet or low-protein diet*

	Diet									
	Full	Low-protein	Full		Low-protein		Low-protein		Low-protein	
No. rats	16	39	5	9	10	9	19	29	10	18
Contraceptive	None	None	A	B	A	B	A	A	B	B
Mode of application	—	—	Vag.	Vag.	Vag.	Vag.	Vag.	Oral	Vag.	Oral
Localization of tumors										
Liver		2			2	1	2	4	3	5
Breast	1	13		1	3	6	9	17	8	9
Skin							1			
Thymus			1	1	5	1	3	2		1
Lymphosarcoma-leukemia					2		2	3	1	1
Lung							3	3	1	
Kidney							2	1		
Stomach					1	1		3		
Intestine		1						1		1
Pancreas								1		2
Uterus		1		1	4	5	3	2	4	3
Brain							1	1		
Pituitary					1	1			2	

present. For example, a rat having mammary tumors in several breasts, which was a rather common occurrence, is tabulated as 1 rat with 1 tumor. If, however, a rat had a tumor in the breast and another in the uterus, it is designated as 1 rat with 2 tumors.

In the control rats on the full diet 1 mammary tumor was found. In the untreated rats on the low-protein diet large numbers of mammary tumors occurred; 2 tumors of the nodular-cholangiofibrotic type were seen; 1 fibrosarcoma of the uterus and 1 in the small intestine (MJ 82) occurred. The tumor of the intestine was an adenocarcinoma that metastasized widely in the mesenteric lymph nodes (fig. 1).

In the first experiment, of the 14 surviving rats on the full diet that were treated with daily vaginal instillations of the contraceptives, 3 rats developed 4 tumors—1 localized in the breast, 1 in the uterus, and 2 in the thymus. This small number of tumors apparently does not exceed significantly the number expected in elderly rats.

In the first group, of the 19 contraceptive-treated rats on a low-protein diet, 16 rats developed 33 tumors, which is far in excess of the number of spontaneous tumors expected. Nine tumors were localized in the breast. This number does not exceed the number of breast tumors in untreated rats on the low-protein diet. The localization of the other tumors is given in table 4. Nine tumors occurred in the uterus (figs. 4, 5, 8, 9). They grew as large polypoid excrescences at the cervix with either smooth or granular surfaces. The size of the tumor varied from a few mm. to about a cm. They all seemed to originate in the vaginal portion of the uterus. On microscopic examination the tumors were

found to consist of fibrous or adenomatous polyps covered by multilayered squamous epithelium. The squamous epithelium often showed metaplastic changes and in one case (fig. 9) showed marked downgrowth. The epithelial cells were large, with large hyperchromatic nuclei and several mitoses, suggesting malignant changes. Three tumors had characteristics of sarcomas, with invasion of the uterine wall, irregular mitoses, giant cells, and marked anaplasia. The seemingly malignant spindle-shaped cells in one instance (fig. 8) were arranged around newly formed vessels, giving the impression of angiofibrosarcoma. The ovaries were not enlarged, but many showed marked stroma luteinization; however, this was present in nearly all rats on the low-protein diet and in some rats on the full diet.

In the majority of rats kept on the low-protein diet the stomach developed squamous metaplasia, squamous papillomas, and ulceration in the forestomach. In 2 rats treated with contraceptives, sarcomas of the stomach occurred. These tumors had the characteristics of fibrosarcoma; they ulcerated the overlying epithelium, and 1 invaded the liver (KC 824) (figs. 12, 13, 14).

A number of tumors in the anterior mediastinum were observed. Eight of these obviously originated in the thymus. Histologically, they consisted of dark, small lymphoid cells and large epithelioid cells with pale-staining nuclei and protoplasm; they were tabulated as thymomas. Two anterior mediastinal tumors were associated with other lymphoid tumors in the lymph nodes and in lung. Histologically, they consisted of immature lymphoid cells and were designated as lymphosarcomas. Two pituitary tumors were found; they were single or multiple adenomas (fig. 2).

Because of the localization of tumors at sites other than the site of application, it was assumed that the rats absorbed and/or ingested some of the material by licking their genitalia. The following experiment was conducted only on rats kept on a low-protein diet, by vaginal administration of the contraceptive substances to one group and by oral instillation in another. Fifty-seven of 76 rats developed tumors; the localization is given in table 4. The number of breast tumors (fig. 3) was slightly higher than in untreated rats kept on a low-protein diet ($P=0.02$–0.05). Although the number of uterine tumors was somewhat lower than in the previous experiment, the gross and microscopic features of the tumors were similar (figs. 6, 7, 10, 11). The gross and microscopic features of thymomas and lymphosarcomas were similar to those observed in the first set of experiments. More tumors at uncommon sites of localization occurred in rats treated with contraceptive A than B, but with the relatively small number of experimental animals the significance of this finding is doubtful.

One rat (KJ 874, contraceptive A, orally) developed a lymphosarcoma that involved, in addition to the lymph nodes and thymus, the stomach, liver, and spleen (figs. 15, 16, 17, 18). The same animal had a fibrosarcoma in the uterus. In another rat (KH 866, contraceptive A, orally) myeloid

leukemia occurred. The spleen of this animal, which died in the 16th month of the experiment, weighed 6.78 gm. Cells of myeloid leukemia infiltrated all organs (figs. 19, 20, 21) and the vessels were filled with myeloid cells. In another rat (KF 849, contraceptive A, vaginally) lymphosarcomatous infiltration was restricted to the spleen.

Two tumors in the stomach were spindle-cell sarcomas (KF 874 and 876). As in the previous experiment with rats on the low-protein diet, many papillomas of the forestomach were observed. In 1 rat (KF 846, contraceptive A, vaginally) the lesion showed marked downgrowth of the hyperplastic epithelium with many mitoses, suggesting early carcinoma.

A tumor of the kidney was found in each of 3 rats treated with contraceptive A. They were located in 1 kidney only; in each instance gross examination showed marked enlargement; the 3 tumorous kidneys weighed 4.8, 3.0, and 2.9 gm., respectively. In 2 instances the tumor replaced the larger part of the kidneys and was located subcapsularly. Microscopically, the lesion consisted, in 2 cases (KG 857, KJ 878), of a tissue of deeply staining small cells arranged in irregular cords and having structures resembling glomeruli. These tumors were thought to be embryonal carcinomas of the kidneys. In the third kidney (KG 853) tumor, the small cells seemed to infiltrate between the glomeruli and in all likelihood represented a lymphoid tumor (figs. 22, 23, 24, 25).

Two brain tumors occurred, 1 in a rat which received contraceptive A orally (KJ 875), the second in an animal treated with contraceptive A by vaginal instillation (KG 854). The brain in both cases was grossly enlarged, and a white, soft enlargement was seen in the region of the basal ganglion (figs. 26, 27, 28, 29). Microscopically, both lesions were moderately poorly differentiated astrocytomas and were composed of large, dark-staining cells resembling fibrous astrocytes. The phosphotungstic acid-hematoxylin stain showed blue-staining glial fibers in the processes of some of these cells. One rat had, in addition to the brain tumor, a tumor in the uterus; the second had no other tumors.

One ulcerated skin tumor in the pre-auricular area was observed in 1 rat (contraceptive A, vaginally). This tumor consisted of large groups of closely packed cells showing the characteristics of a lymphoid tumor. Two pancreatic and 2 pituitary adenomas developed in the group treated with contraceptive B and 1 pancreatic adenoma in those given contraceptive A. The pituitaries in many other animals were enlarged; however, on histologic investigation no circumscribed lesion was found.

Discussion

In the interpretation of the results obtained by prolonged application of spermicidal contraceptives to rats, 3 major factors must be considered: 1) the effect of aging, 2) the effect of diet, and 3) the effect of the contraceptives.

The frequency with which spontaneous tumors occur in rats increases with age. Bullock and Curtis (*1*) found 489 tumors in 2,450 rats whose original source is apparently unknown. Curtis *et al.* (*2*) analyzed the

occurrence of spontaneous tumors in 7 different strains and found that in all strains 2.14 percent of the females were tumor bearers. In the 14-to-18-month age group 1.22 to 2.57 percent of the rats found dead each month were tumor bearers. Arai (*3*) observed 27 spontaneous tumors in 500 rats of different strains. In the present experiment the control and treated groups fed full diets showed incidences of tumors similar to those quoted above.

The effect of diet on tumor production, with respect to both caloric restriction and variation of composition (proteins, fat, and vitamins), has been extensively investigated and was reviewed recently (*4*). Relevant to the present investigation are the facts that certain low-protein diets induce liver damage and that the activity of at least 1 well-known carcinogen, *N,N*-dimethyl-*p*-phenylazoaniline (*p*-dimethylaminoazobenzene) depends on dietary factors. The diet employed in the experiments presented was moderately low in proteins. The protein used was casein purified by hot alcohol. The effect of the procedure in rendering the casein vitamin-free must be considered. Liver damage is less readily produced by alcohol-purified casein than, *e.g.*, by alkali-extracted casein (*5*). Whether a specific amino acid deficiency, in addition to the over-all effect of a low-protein diet, might have been effective in tumor production is at present being investigated.

The experiment concerned with the effect of the purified low-protein diet alone showed that the diet causes liver damage in all the rats; in addition, a large number of breast tumors occurred. In all likelihood, the 2 findings may be interrelated; the damage to the liver impairs the detoxication of the estrogens, which in turn, act as a carcinogen for the breast. Apart from tumors of the breast and liver, 1 intestinal and 1 uterine tumor were found in these rats. In table 3 the liver and breast tumors, which in all likelihood developed as a consequence of dietary deficiency, are separated from other tumors, for which such an etiology is not assumed.

The substance or substances responsible for the increased number of tumors in rats treated with contraceptives is not known at present. Of interest are the unusual types of tumors that occurred. Brain tumors remote from the site of the application of the carcinogenic substance in rats have been reported only twice; these tumors occurred in rats fed *N*-2-fluorenylacetamide (2-acetylaminofluorene) (*6,7*). Dr. H. L. Stewart (personal communication) saw 1 brain tumor in an untreated rat.

Myelogenous leukemia is also uncommon in rats. Shay *et al.* (*8*) in 1951 summarized the findings reported previously and described 6 cases of lymphatic and 2 cases of myelogenous leukemia in their experiments.

Bullock and Curtis (*1*) found 8 tumors in the kidneys, 6 of which were designated as embryonal carcinoma. The description of these tumors agrees in all details with that of tumors in the present material.

Contraceptive A contains 8-*ortho*-hydroxyquinoline, and the carcinogenicity of this substance is now under investigation. Recently, Boyland and Watson (*9*) reported that instillation of cholesterol pellets containing 20 percent 8-*ortho*-hydroxyquinoline into the bladder of mice resulted in production of papillomas and carcinomas.

To what extent the findings described can be related to the problems of cervical carcinoma in humans cannot be answered at present. Several features that render unusually difficult the application of the experimental findings to the human problem should be pointed out: First, the uterine tumors that occurred in rats treated with contraceptives were mostly of connective-tissue origin; in humans, malignant connective-tissue tumors of the uterus are rare. It should, however, be kept in mind that rats develop sarcomas more frequently than carcinomas. The effect of the same carcinogens might be different in different animal species. Second, the possibility that the contraceptive material, which was ingested, exerted carcinogenic activity even after intravaginal application cannot be eliminated. The quantity of active substances absorbed through the vaginal mucosa could not be established. Third, it appears that dietary imbalance with damage of the liver was a most important requirement for the carcinogenic activity of the substance, or substances, present in the contraceptives. Such a condition may not be present in many groups of the human population; in other groups, however, severe nutritional liver damage is a common occurrence. Whether the impairment of detoxication of estrogens, which occurs under such circumstances, may enhance the carcinogenic activity of the substance present in contraceptives is being investigated.

Summary

Two spermicidal contraceptives were investigated for carcinogenic activity in rats. The contraceptives were administered either by vaginal instillation or by oral feeding to rats kept on a full diet or on a purified diet that had a low protein content.

The 39 control rats kept on the low-protein diet developed liver damage, and 13 had mammary tumors. One uterine and 1 intestinal tumor were observed.

In the 14 rats fed the full diet and treated with the contraceptives the number of tumors did not exceed the number expected in aging rats.

In 2 separate experiments 73 of 95 rats kept on the low-protein diet and treated with contraceptives, either by vaginal or oral administration, developed tumors. In addition to mammary and liver tumors, which occurred also in untreated rats kept on the same diet, 69 tumors occurred at other sites. The large number and unusual localization of the tumors (*e.g.*, 2 in brain) renders extremely small the probability that the results are due to chance.

The chemical substance responsible for tumor production is not yet known. The carcinogenic activity of one constituent of contraceptive A, 8-*ortho*-hydroxyquinoline, is now under investigation.

The implication of the findings relative to the problem of cervical carcinoma in human beings is discussed.

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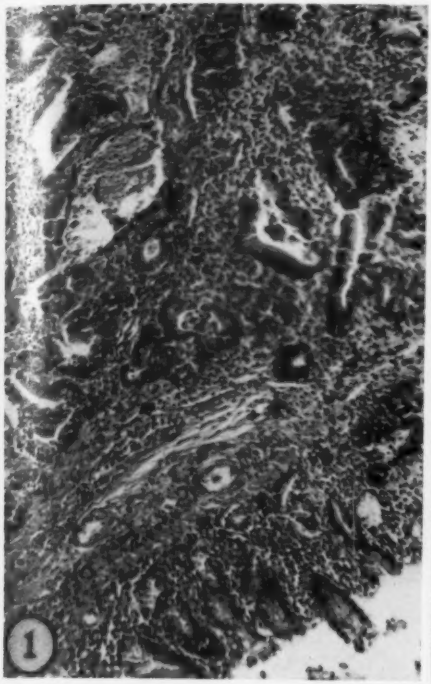
All microscopic sections stained with hematoxylin and eosin

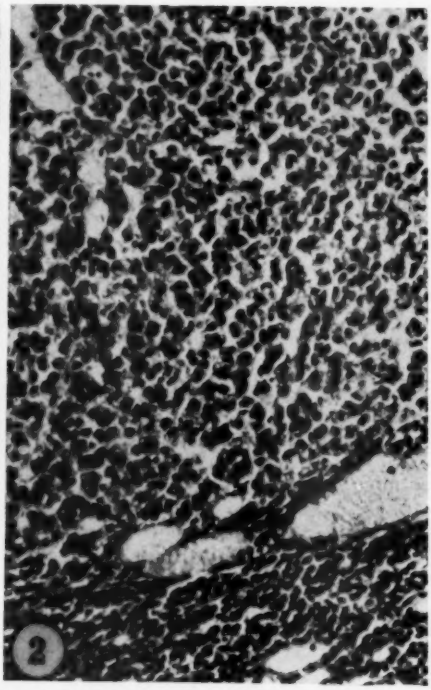
Plate 43

Figure 1.—*Adenocarcinoma of small intestine, MJ 82, low-protein diet.* The *lowe right* corner shows the normal mucosa of the small intestine. The submucosa and muscularis are invaded by adenocarcinoma. × 120

Figure 2.—*Adenoma of pituitary, KC 822, contraceptive B, vaginally.* At the *bottom*, part of the normal pituitary is visible. The adenoma is well-demarcated from this tissue and consists of large cells with abundant cytoplasm and large, pale-staining nuclei. × 200

Figure 3.—*Tumor in breast, KH 865, contraceptive A, orally.* The tumor is arranged in alveolar structures invading the stroma; the alveolar structures are lined with multilayered, irregular epithelium. × 385





Hoch-Ligeti 671

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PLATE 44

Gross appearance of uterine tumors

FIGURE 4.—*KC 828, contraceptive B, vaginally.* Large polypoid mass originating from the cervix. The vagina is markedly dilated. × 1.6

FIGURE 5.—*KD 835, 836, 837, contraceptive A, vaginally.* Three cervical tumors of different sizes. The larger globular structure in KD 837 had a cauliflowerlike appearance. (For microscopic diagnosis *see* fig. 9.) × 0.9

FIGURE 6.—*KG 859, contraceptive A, vaginally.* Large, hard tumor involving cervix and posterior part of vaginal vault. (For microscopic diagnosis *see* fig. 11.) × 1.6

FIGURE 7.—*KO 976, contraceptive B, orally.* Large tumor originating in the left uterine horn and involving the entire wall. × 0.8

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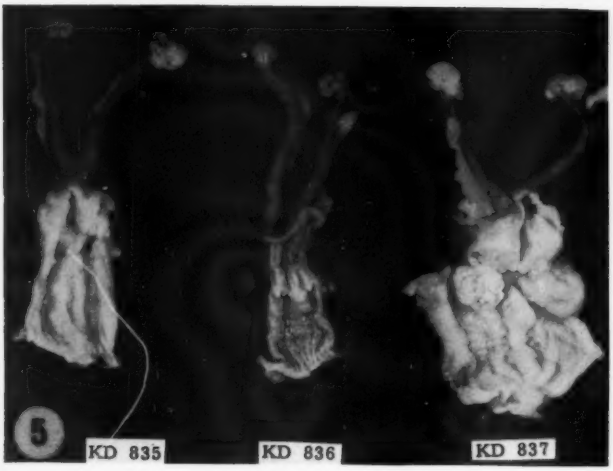
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KD 835
KD 836
KD 837

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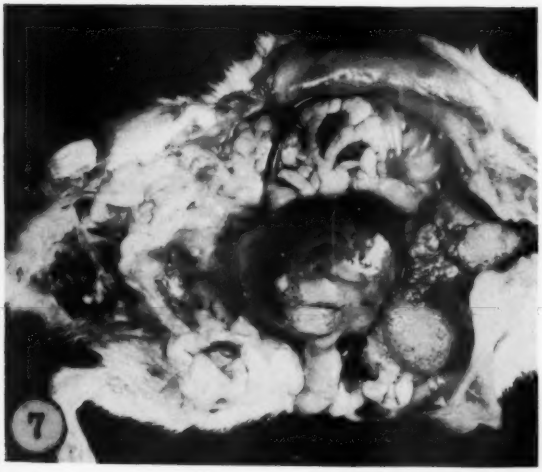
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PLATE 45

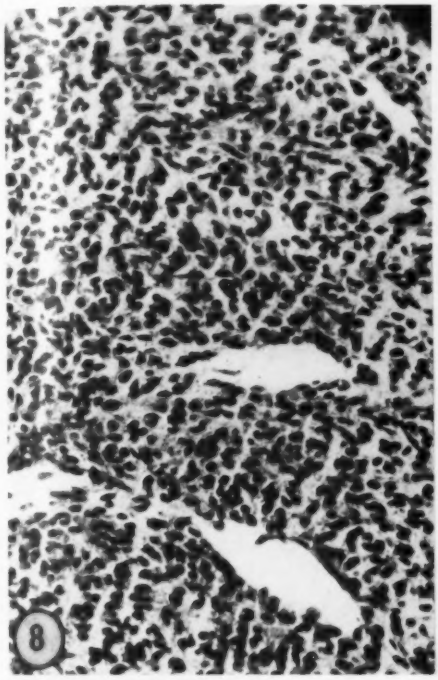
Microscopic appearance of uterine tumors

FIGURE 8.—*Angiofibrosarcoma, KC 822, contraceptive B, vaginally.* The tumor is composed of large spindle-shaped cells arranged around and apparently arising from vessels. × 300

FIGURE 9.—*Epidermoid carcinoma of cervix, KD 837, contraceptive A, vaginally.* Large, irregular squamous epithelial cells invading the cervical stroma. Numerous mitotic figures are present. × 200

FIGURE 10.—*Adenomatous polyp of endometrium, KG 857, contraceptive A, vaginally.* The tumor is composed of irregular glands with sparse stroma. Every gland has a definite basal membrane. × 175

FIGURE 11.—*Fibroma of cervix, KG 859, contraceptive A, vaginally.* The tumor is composed of fusiform cells with dense intracellular fibrils and shows a whorl-like arrangement. × 200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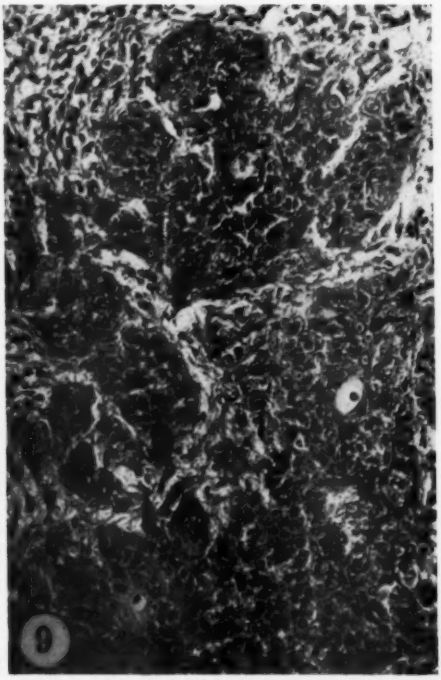
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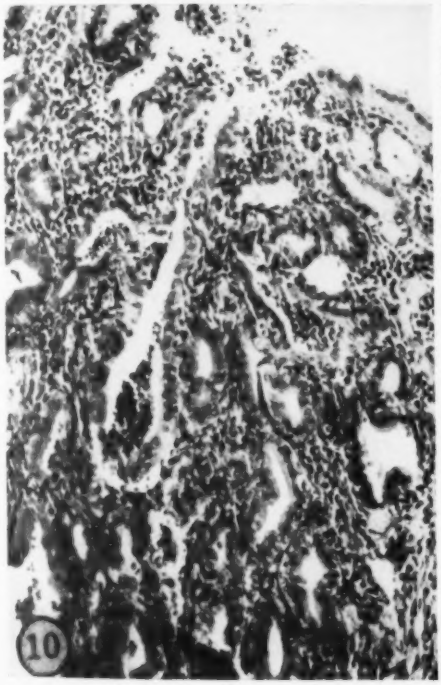
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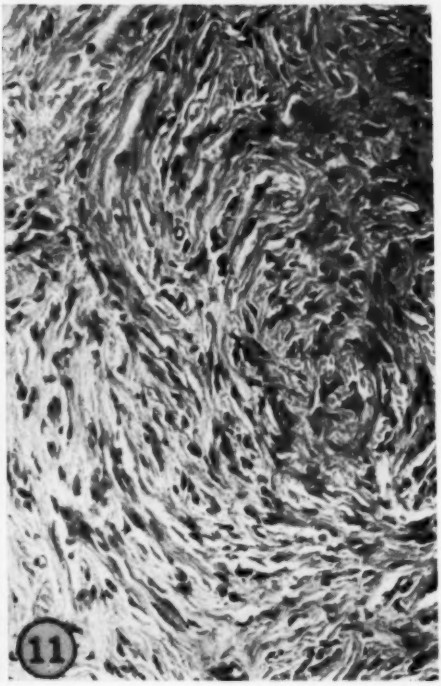
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PLATE 46

Lymphosarcoma of the stomach in rat KC 824 (contraceptive B, vaginally)

FIGURE 12.—*Stomach.* The mucosa of the stomach is markedly thickened, with several small tumors and 1 large tumor, which occupies nearly the whole forestomach. × 1.6

FIGURE 13.—*Stomach tumor (same as fig. 12).* The entire submucosa is invaded by lymphosarcoma, containing both small, dense, deeply stained cells, and large cells with pale nuclei. × 400

FIGURE 14.—*Lymphosarcoma invading liver.* The *lower left* corner shows strands of liver cells separated by tumor cells with the same histologic characteristics as in figure 13. × 400

KC824
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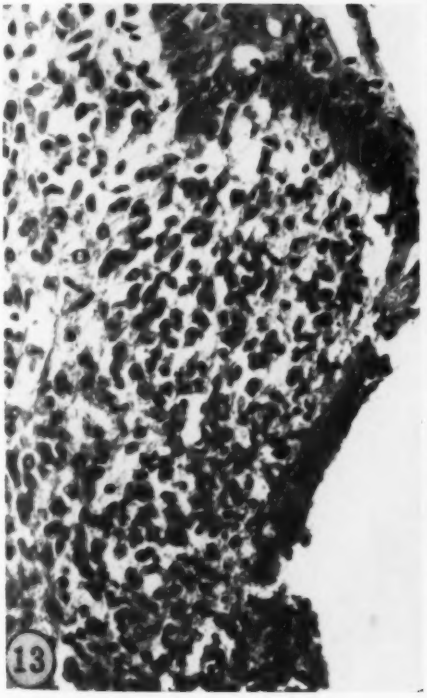
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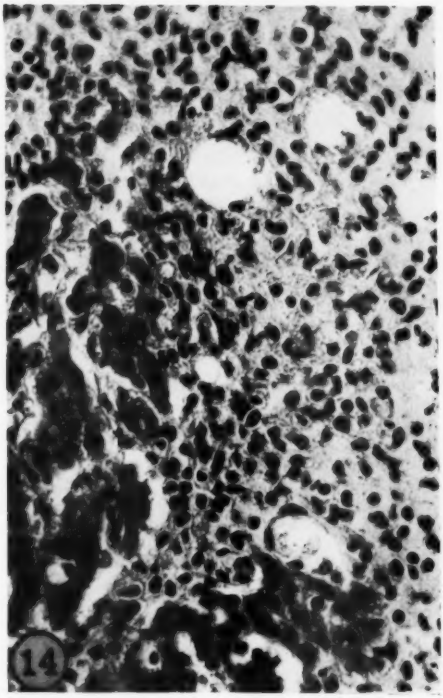
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PLATE 47

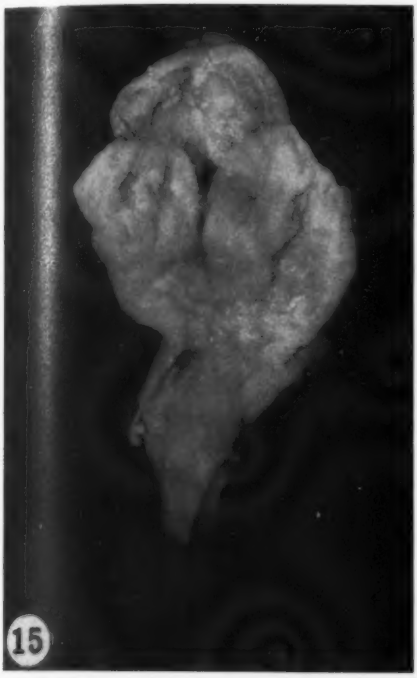
Tumors in rat KJ 874 (contraceptive A, orally)

FIGURE 15.—*Lymphosarcoma of stomach.* The wall of the entire glandular stomach is diffusely thickened, with marked narrowing of the lumen and no ulceration of the mucosa. × 1.6

FIGURE 16.—*Lymphosarcoma in stomach.* The *upper left* corner shows the glandular mucosa. The entire wall of the stomach is infiltrated by dense lymphoid tissue consisting of small, dark-staining cells. × 50

FIGURE 17.—*Lymphosarcoma invading around adrenal.* The cells are round, with hyperchromatic nuclei and sparse cytoplasm. × 700

FIGURE 18.—*Fibrosarcoma of uterus.* The uterine tumor is composed of large spindle-shaped cells arranged in irregular bundles. × 350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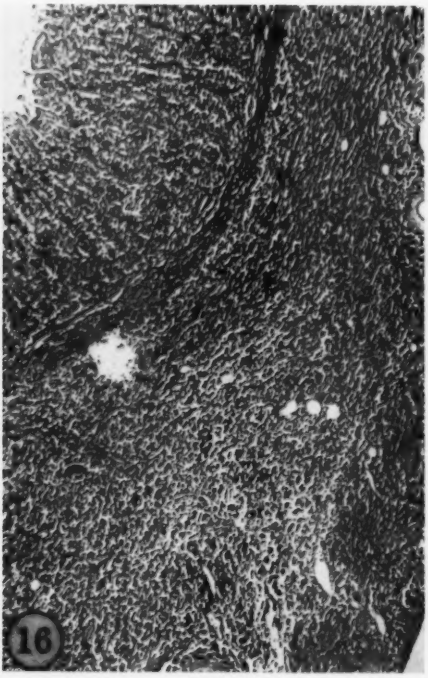
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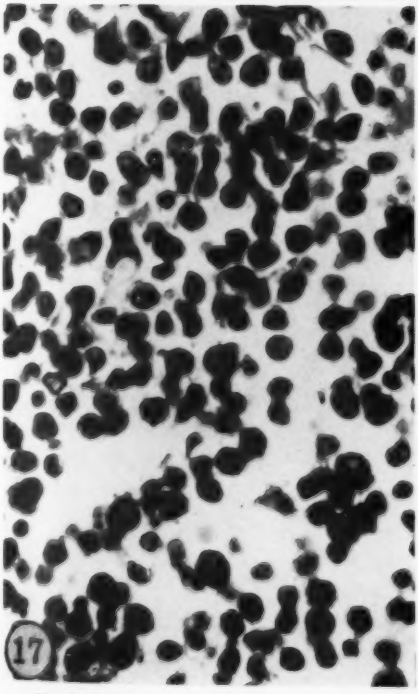
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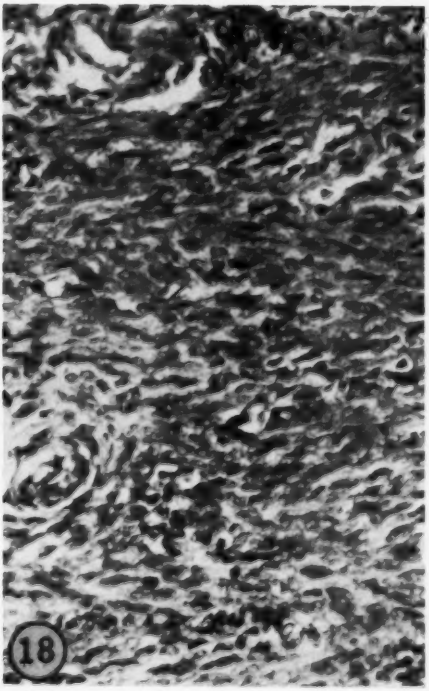
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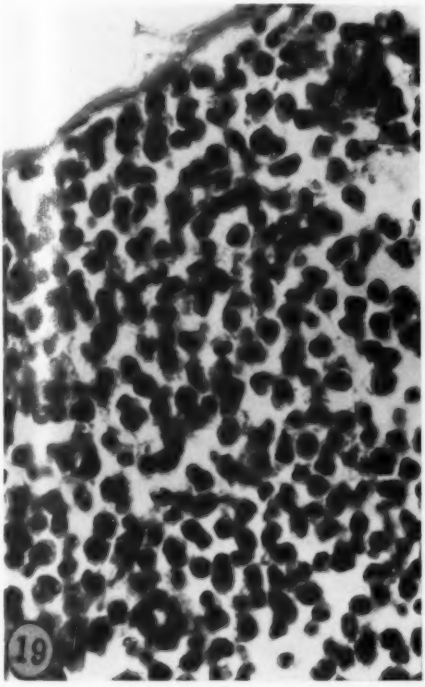
PLATE 48

Myeloid leukemia in rat KH 866 (contraceptive A, orally)

FIGURE 19.—*Lymph node* showing destruction of structure by myeloid infiltration. Most of the cells are large and have lobulated nuclei. × 650

FIGURE 20.—Infiltration of lung and vessel filled with myeloid cells. × 325

FIGURE 21.—*Myeloid infiltration of kidney.* The perivascular space between glomeruli is densely infiltrated with myeloid cells. The infiltration also extends between the tubules. × 300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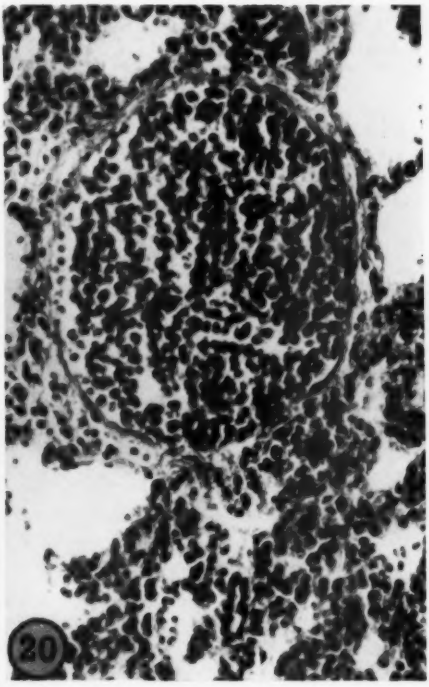
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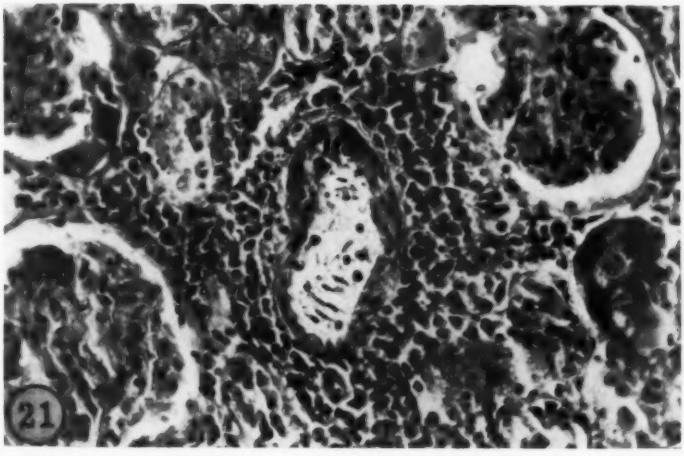
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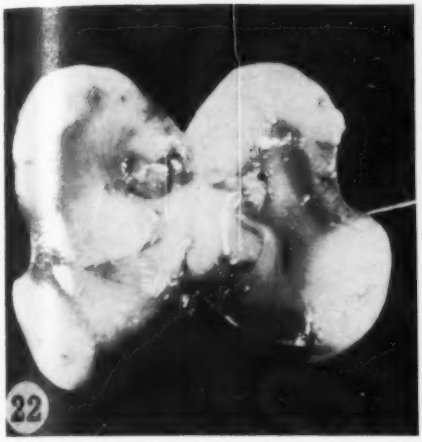
PLATE 49

FIGURE 22.—*Kidney tumor in rat KG 857, contraceptive A, vaginally.* The kidney is enlarged to 4.88 gm. The structure of the upper and part of the lower pole is replaced by a white mass that projects over the cut surface. × 1.9

FIGURE 23.—*Kidney tumor (same as fig. 22).* Embryonal carcinoma of kidney. The tumor is composed of irregular large cells with hyperchromatic nuclei and dark-staining cytoplasm. Structures resembling tubules are present. × 400

FIGURE 24.—*Kidney tumor, KJ 878, contraceptive A, orally.* Irregular small tumor cells infiltrating between glomeruli. × 400

FIGURE 25.—*Embryonal carcinoma of kidney, KG 853, contraceptive A, vaginally.* The tumor is composed of masses of dark-staining, large cells and shows new formation of atypical small glomeruli and tubules. × 470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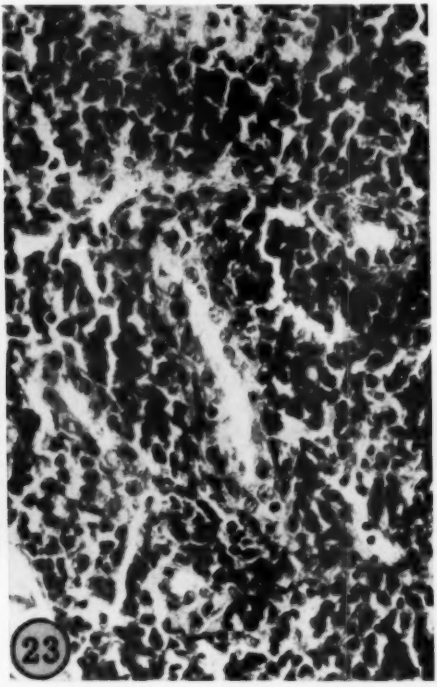
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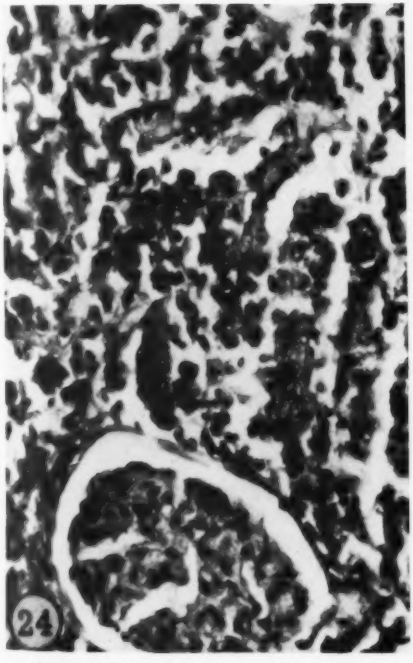
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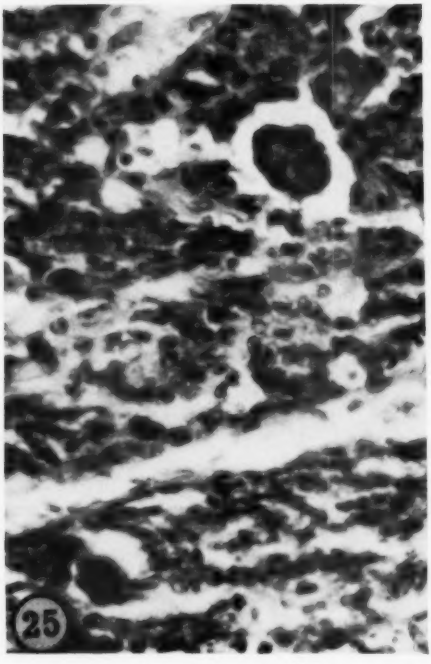
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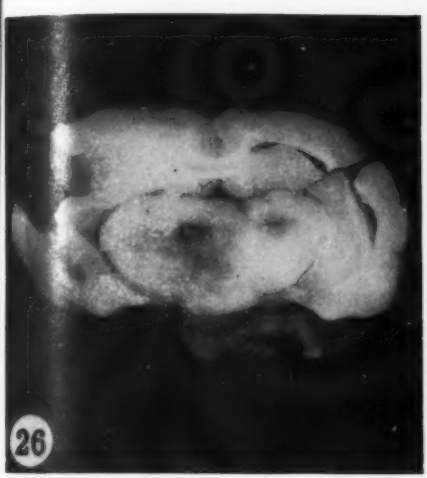
PLATE 50

FIGURE 26.—*Brain tumor, KJ 875, contraceptive A, orally.* The white tumor enlarges the thalamus on the *right* side of the photograph. × 3

FIGURE 27.—*Brain tumor (same as fig. 26).* Glioma. At the *right* are the cortex of the hippocampus, lateral ventricle, and choroid plexus. The tumor invades from the thalamus on the *left* to beneath the ependyma on the lateral as well as the medial (left) side of the ventricle. × 65

FIGURE 28.—*Brain tumor (same as fig. 26).* The tumor is composed of elongated cells resembling astrocytes with dark nuclei and interlacing fibrils. × 700

FIGURE 29.—*Brain tumor, KG 854, contraceptive A, vaginally.* Glioma. This tumor, like the previous one, contains elongated astrocytic cells with dark nuclei and interlacing fibrils. × 700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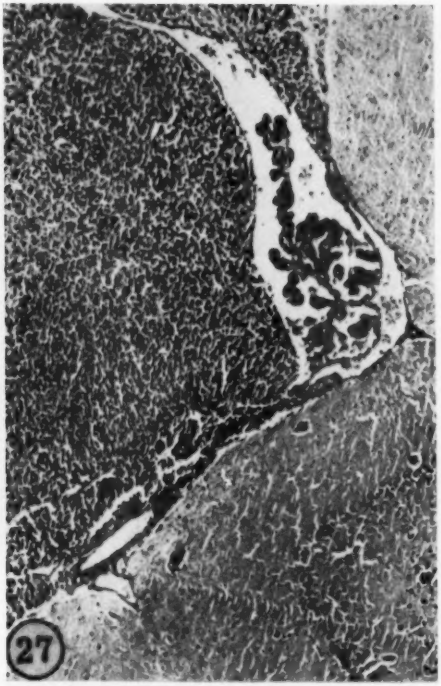
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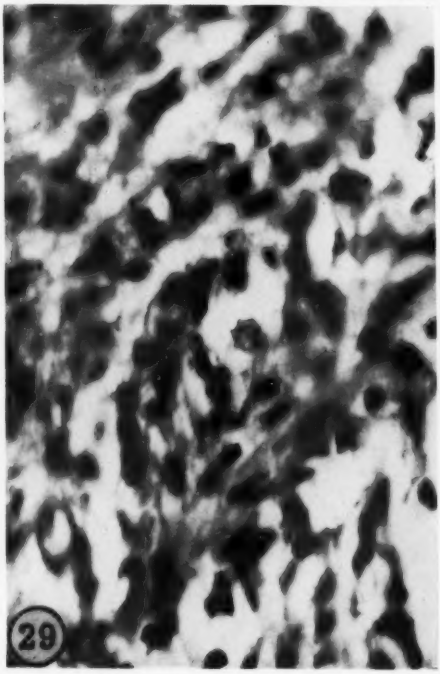
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A Study of the Chemical Constitution and Carcinogenic Action of Creosote Oil [1, 2]

WILLIAM LIJINSKY, UMBERTO SAFFIOTTI,[3] *and* PHILIPPE SHUBIK, *Division of Oncology, The Chicago Medical School, Chicago, Illinois*

Creosote is a widely used material that has been implicated as an environmental carcinogen in man (*1–3*). Sall and Shear (*4*) demonstrated that the basic fraction of creosote, although noncarcinogenic, could augment skin carcinogenesis induced in the mouse by a dilute solution of benzo[*a*]pyrene. This phenomenon was termed cocarcinogenesis. Several other substances have been found to have a cocarcinogenic action and, among these, the most potent was croton oil (*5*). In the original studies these agents were applied concurrently with a weak solution of a carcinogen; subsequently it was found that croton oil could complete carcinogenesis when applied following a single, subeffective dose of carcinogen (*6*). This activity, under these conditions, has been termed "promoting action" (*7*). In the present study the possible promoting activity of creosote and its basic fraction has been investigated, and a chemical analysis of the neutral fraction has been undertaken to determine the nature of its active constituents.

Materials and Methods

The creosote chosen for this study was a #1 oil, dry point 240° C., collected as a distillation fraction from a Wilton still. The creosote was first tested undiluted, then as a 10 percent solution of the whole material in acetone, and finally as a 2 percent solution of the basic fraction in acetone. The basic fraction was prepared by the procedure of Cabot *et al.* (*8*).

Swiss female mice, originating from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, were used and subsequently bred in this laboratory. Treatments consisted of applying 1 drop of solution with a glass dropper to the interscapular region of skin, which was kept free of hair by clipping with scissors. All tumors that appeared were charted

[1] Received for publication January 16, 1957.

[2] This investigation was supported by research grant CS-9212 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and research grant ENV-7 from the American Cancer Society, Inc.

[3] *Present address:* University of Milan, Milan, Italy.

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weekly according to a scheme previously described (*9*) and were examined histologically.

Experiments and Results

Biological

Four groups of 30 Swiss female mice each were used in this study. Group 1 was painted twice weekly with undiluted creosote. Group 2 received a single initial painting of 1.0 percent 7,12-dimethylbenz[*a*]anthracene (DMBA) (Eastman Organic Chemicals) in mineral oil (Superla 34, Standard Oil of Indiana), and beginning 1 week later was treated with undiluted creosote twice weekly (as group 1). Group 3 received a single application of DMBA and was subsequently treated with 10

TABLE 1.—*Results of 4 schedules of treatment with creosote on Swiss female mice*

Group	Treatment*		No. of mice	No. survivors at time of first tumor	No. of tumor-bearing mice	Total no. of tumors†	No. of carcinomas	Average latent period (*weeks*)
	Single painting	Painting twice weekly						
1	—	Creosote: undiluted	30	26	13	23	16	50
2	1% DMBA	Creosote: undiluted	30	23	17	32	26	39
3	1% DMBA	Creosote: 10% in acetone	30	29	11	15	8	43
4	1% DMBA	Creosote basic fraction: 2% in acetone	30	12‡	—	—	—	—

*One-week interval between the initiating single application and beginning of the creosote paintings.
†No tumor regressed.
‡Survivors at 56 weeks.

percent creosote in acetone. The mice in group 4 received a single application of DMBA and were subsequently painted twice weekly with a 2 percent solution in acetone of the basic fraction of the creosote. A control group of 50 Swiss female mice received a single application of 1.0 percent DMBA in mineral oil and no other treatment. No tumors occurred in the controls after an 80-week observation period.

The results of the experiment are recorded in table 1. The experiment was continued for 70 weeks, at which time the survivors were sacrificed and autopsied. All treatments were continued throughout the experiment. From table 1 it can be seen that only the undiluted creosote proved to be carcinogenic (group 1) and that, when applied following a single initiating dose of DMBA, some augmentation of the action could be noted (group 2). With a dilution of the creosote to 10 percent in acetone, the action was somewhat diminished (group 3), but the basic fraction of

this creosote applied as a promoting agent (group 4) did not possess any activity. It is of some interest to compare these results with an experiment run in this laboratory at the same time and published elsewhere (*10*) in which the action of a 0.01 percent solution of DMBA in mineral oil was studied under conditions identical with those used for the undiluted creosote in groups 1 and 2 of this study. It was found that repeated applications of 0.01 percent DMBA induced tumors in 10 of 20 treated mice. A total of 27 tumors, 13 of them malignant, was observed after a latent period of 50 weeks. With the undiluted creosote, 13 of 26 treated mice developed 23 tumors, again with a latent period of 50 weeks; of these 16 proved malignant. When 0.01 percent DMBA was applied to skin previously treated only once with an application of 1.0 percent DMBA, 17 of 30 treated mice developed a total of 47 tumors, of which 24 were carcinomas; the latent period was reduced to 43 weeks. When the undiluted creosote was applied following a single application of carcinogen, 17 of 30 treated mice developed a total of 32 tumors, of which 26 proved to be malignant; the latent period was reduced to 39 weeks. Thus, the undiluted creosote proved to have a biological action almost identical with that of a 0.01 percent solution of DMBA in mineral oil.

Chemical

The method of analysis used was a combination of chromatography and ultraviolet spectrophotometry in 2,2,4-trimethylpentane (using a Beckman DK-1 instrument). The adsorbent was a 2:1 mixture of magnesia-celite [4] and the eluents were mixtures of hexane-benzene-acetone.

The initial treatment of the creosote involved preparation of a neutral fraction that was most likely to contain the carcinogenic constituents (the basic fraction was noncarcinogenic). One liter (1030 gm.) of creosote was mixed with 800 ml. of hexane and 300 ml. of benzene and poured away from the small amount of insoluble tar. The solution was shaken for 24 hours with 2 successive 1500 ml. portions of 30 percent sulfuric acid, followed by 2 1500 ml. portions of 20 percent sodium hydroxide. After washing, the solvent layer was evaporated under nitrogen, leaving 742 gm. of oil, which largely solidified on cooling.

This material was steam-distilled, giving 73 gm. of distillate (containing naphthalene). The nonvolatile residue was filtered in 2 portions through 100 gm. of magnesia-celite, which retained much fluorescent material. After elution with the ethanol-acetone-benzene mixture and evaporation, the eluate weighed about 80 gm. Further chromatography resulted in the isolation of the major constituents of the mixture in pure crystalline form. They were, in order of decreasing adsorption affinity: carbazole, benz[*a*]anthracene, chrysene, fluoranthene, pyrene, anthracene, and phenanthrene. They were identified by means of the absorption spectra, melting points, and preparation of the 2,4,7-trinitrofluorenone complexes of these compounds. No benzo[*a*]pyrene or other known carcinogen was

[4] Magnesia—Seasorb 43, Food Machinery & Chemical Corporation; Celite No. 45—Johns-Manville Co.

isolated, although the possibility could not be excluded that such substances might be present in quantities too small to be isolated.

A quantitative analysis of the creosote was performed, using an aliquot of the neutral fraction. On a large column of adsorbent, 0.63 gm. was chromatographed and after adequate development (during which most of the anthracene and phenanthrene passed into the filtrate), all the fluorescent zones were eluted, their spectra were read, and the quantity of each constituent was assayed by the extinction at some absorption maximum. Benzanthracene and chrysene were in the same chromatographic zone, as were fluoranthene and pyrene, and also anthracene and phenanthrene. The quantity of each constituent was estimated by difference spectra, with extinction values obtained from Clar (*11*). The results of this analysis are given in table 2.

TABLE 2.—*Analysis of creosote constituents*

Creosote constituents	Volume of solution (*ml.*)	ε (max. mμ)	Concentration in creosote (*gm./l.*)	
Carbazole	82	2. 70 (291)	2. 75	
Benzanthracene	78	10. 90 (288)	2. 75	
Chrysene	78	9. 20 (267)	1. 27	
Fluoranthene	245	5. 32 (288)	7. 8	
Pyrene	245	3. 20 (334)	4. 2	
Anthracene				
Adsorbed	255	5. 43 (252)	1. 4	6. 2
Filtrate	2920	1. 60 (252)	4. 8	
Phenanthrene				
Adsorbed	255	1. 65 (293)	5. 7	47. 9
Filtrate	2920	1. 08 (293)	42. 2	

To determine the pentacyclic compounds in the sample, a larger quantity was chromatographed and only the uppermost zone was examined (the remainder being allowed to run into the filtrate). Thus, 2.35 gm. of neutral fraction gave such a zone, the spectrum of which had well-defined absorption maxima at 350 and 359 mμ (indicative of pentaphene) as well as an inflection at 384 mμ (indicative of benzo[*a*]pyrene). After correction for general absorption, the extinctions at these wavelengths corresponded to concentrations of 70 mg. per l. of pentaphene and 40 mg. per l. of benzo[*a*]pyrene.

No fraction rich in benzo[*a*]pyrene could be isolated, although several had the 384 mμ absorption which may be attributed to this compound. One large fraction showing this band consisted largely of carbazole. The relative intensities of the 291 mμ band (due to carbazole) and the 383 mμ band were such that the maximum benzo[*a*]pyrene content of this fraction was somewhat less than 100 mg. (from 1 liter of creosote).

The characteristic fluorescence spectrum of benzo[*a*]pyrene was considered indicative only of the presence of this compound, since no quantitative relationship could be established between the fluorescence maxima and concentration of benzo[*a*]pyrene.

A more accurate determination of the benzo[*a*]pyrene content of creosote was now made. A 30 ml. aliquot of creosote was subjected to chromatographic analysis, and the fractions were examined fluorimetrically for benzo[*a*]pyrene. Those fractions showing benzo[*a*]pyrene fluorescence were combined and chromatographed further until a single fraction was obtained in which the benzo[*a*]pyrene could be assayed in the ultraviolet spectrophotometer. A similar aliquot of creosote to which benzo[*a*]pyrene had been added in the proportion of 100 mg. per l. of creosote was processed in an identical manner and the benzo[*a*]pyrene in the final fraction containing the compound was estimated. The difference in the 2 determinations was equal to 70 percent of the added benzo[*a*]pyrene, which was an adequate recovery when the losses involved in chromatography are considered.

The benzo[*a*]pyrene assay was made at 384 mμ, the extinction at this wavelength having been corrected for general absorption. The maximum benzo[*a*]pyrene content of the creosote was thus calculated as 120 mg. per l. However, because of the presence of a compound with λ max. 380 mμ [possibly benzo[*k*]fluoranthene (*11*)], which contributed materially to the absorption at 384 mμ, the true benzo[*a*]pyrene content must be rather less than 100 mg. per l. of creosote. Also present in the fraction containing benzo[*a*]pyrene (and identified by the absorption maxima) were perylene, pentaphene, and (probably) benzo[*e*]pyrene, as well as benzo[*k*]fluoranthene. The concentrations of these compounds was approximately the same as that of benzo[*a*]pyrene (*e.g.*, perylene concentration, calculated at 437 mμ, was 40–60 mg./l.). Also identified in the mother liquors of the benz[*a*]anthracene crystallization was triphenylene. No hexacyclic compounds could be identified in the creosote fractions.

Discussion

In view of the cocarcinogenic activity of a basic fraction of creosote, demonstrated by Sall and Shear (*4*), a sample of this material was tested for promoting action. Unfortunately, the sample of creosote we studied did not show this type of activity, but it was found to be carcinogenic. Although the sample of creosote was found to yield biological results comparable to those found with a 0.01 percent solution of the potent carcinogen 7,12-dimethylbenz[*a*]anthracene in mineral oil, no known carcinogen that could account for this was found. If this sample does indeed contain benzo[*a*]pyrene, then it is certainly present in amounts far too low to account for the biological activity demonstrated. A relatively large quantity of benz[*a*]anthracene (2.75 gm./l.) was found. This hydrocarbon has been claimed by some workers to be noncarcinogenic or only weakly so (*12,13*), whereas others have found it to be relatively carcinogenic (*14*). It seems possible that benz[*a*]anthracene may be responsible for some of the activity. It is of particular interest that this coal-tar product demonstrates carcinogenic activity that undoubtedly cannot be attributed to benzo[*a*]pyrene.

Summary

Creosote oil was found to have a carcinogenic potency on mouse skin corresponding to a 0.01 percent solution of 7,12-dimethylbenz[*a*]anthracene in mineral oil. Fractionation of the creosote demonstrated the presence of 2.75 gm. per liter of benz[*a*]anthracene. The amounts of benzo[*a*]pyrene present were not adequate to account for the biological activity noted.

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Biliary-Pigment Changes Resulting from Azo-Dye Administration[1,2]

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Bile-duct or oval-cell proliferation is one of the interesting early changes that results from the administration of the carcinogenic azo dyes. Although it still remains to be established that hyperplasia definitely arises from bile-duct epithelium, all the evidence points to this origin (*1*). The problem considered here is how and why this proliferation takes place in the fulminant manner observed. Liver morphology is essentially normal during the first 2 weeks of 3′methyl-4-dimethylaminoazobenzene (3′Me-DAB) feeding, while in the 3rd and 4th weeks bile-duct cells increase over 100-fold in many cases (*2,3*). During the latter period microscopic examination of a few liver sections is sufficient to impress one with the dramatic nature of this change in liver morphology.

Because of the similarity of these changes to those that precede human liver cancer, the South African investigators have shown the most interest in this aspect of liver carcinogenesis. Gillman *et al.* (*4*) developed various surgical and dietary techniques in order to produce changes in liver morphology similar to those resulting from azo-dye treatment. Complete or partial occlusion of the common duct and the feeding of a fat-free diet lacking vitamin A were the most successful methods of eliciting hyperplasia of intrahepatic bile ducts. Previous work concerning the chemical changes that result from bile-duct occlusion caused the authors to suggest that a disturbance in the metabolism of cholic acid or cholesterol might play an important part in promoting bile-duct proliferation under these various conditions. Similar considerations in this laboratory led to the following examination of bile constituents during the course of azo-dye carcinogenesis in rats.

Methods

Three studies were carried out in which male albino rats of the Holtzman-Sprague-Dawley strain were maintained on a purified diet (*5*) containing 0.06 percent 3′Me-DAB. Control groups of rats were also

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maintained on the purified diet alone for comparable periods. Food and water were administered *ad libitum*. After various time intervals, depending upon the experiment, the rats were decapitated and exsanguinated, and samples from different lobes of the liver were placed in 10 percent formalin for histologic examination. The latter was carried out on paraffin-embedded tissues, cut at 6 μ, and stained with hematoxylin and eosin. In the first 2 experiments, the extent of bile-duct proliferation was estimated roughly and graded on an arbitrary basis. In the 3rd experiment, the Chalkley technique (*6*) was used to estimate the percentage of bile-duct cells. The values expressed were obtained from the examination of 60 medium-power fields from 3 different areas of the liver.

Serum bilirubin was determined by a modification of the van den Bergh reaction (*7*), in which the azo coupling was carried out in acid-aqueous solution and the resulting optical density obtained at 570 mμ after 30 minutes. The results were expressed as optical-density units per ml. of serum.

Total serum cholesterol was determined by Delsal's procedure (*8*), and the concentrations were expressed in mg. per 100 ml. of serum. Immediately after heart puncture, hemoglobin was determined on the whole blood by a routine clinical procedure involving the spectrophotometric measurement of oxyhemoglobin in dilute ammonium hydroxide solution. The results were expressed in gm. per 100 ml. of blood.

Fecal-urobilin determination was performed on 2-day fecal collections by a modification of the procedure of Legge (*9*). Feces were allowed to stand with 2 ml. of glacial acetic acid for 30 to 60 minutes and were then ground in an all-glass tissue grinder with additions of ethyl ether. The suspension was separated by centrifugation and the solid material was washed twice with ether. The combined acetic acid-ether extract, which amounted to approximately 30 ml., was washed once with 10 ml. of half-saturated sodium acetate and twice with 10 ml. of distilled water. The combined washings contained the bulk of fecal urobilin, an aliquot of which was added to 10 ml. of a saturated solution of zinc acetate in ethanol. The zinc-urobilin complex which was formed gave a strong green fluorescence that was determined in the fluorimeter with an appropriate filter, using fluorescein as the standard. Results were expressed as ratios of the excretion obtained from rats fed a basal diet, arbitrarily allowing the latter to equal 1.

In experiment 1, rats with an average initial weight of 160 gm. were sacrificed after periods of 2, 4, 8, 16, 29, 38, 46, 63, and 90 days on the 3′Me-DAB diet. Fecal urobilin was examined over the entire period and at sacrifice serum bilirubin and blood hemoglobin were determined. Although an examination of urinary urobilin was attempted, a practical method of estimation was not developed because of the extensive contamination by azo dyes in the urine samples.

In experiment 2, rats with an average initial weight of 195 gm. were sacrificed after treatment with 3′Me-DAB for periods of 0 to 38 days. Serum-bilirubin and serum-cholesterol concentrations were examined

along with concurrent histologic changes in the liver. In experiment 3, the same type of study was carried out over a period of 0 to 26 days in rats with an average initial weight of 171 gm. Weight gains of approximately 100 gm. took place in the control animals, while the body weights of the dye-fed rats tended to remain roughly constant.

Results

From the onset of the fecal-urobilin study, it was observed that rats fed azo dyes demonstrated a marked increase in the excretion of this pigment (table 1). This was apparently the result of an increased hemoglobin breakdown due to the hemolytic activity of either the dye or its detoxication products. *p*-Aminophenol, an active hemolytic agent (*10*), is known to be excreted by rats fed DAB, and it is quite likely that similar compounds are produced from 3'Me-DAB. This hemolysis does not appear to be involved in carcinogenesis, since even more pronounced increases in fecal urobilin were observed when the noncarcinogenic dye 4-aminoazobenzene was fed at the level of 0.08 percent in the diet. Al-

TABLE 1.—*Bile pigment and hemoglobin concentrations in rats ingesting 3'methyl-4-dimethylaminoazobenzene**

Group	Time (*days*)	No. of rats	Serum bilirubin†	Fecal urobilin	Blood hemoglobin (*gm./100 ml.*)	Bile-duct proliferation
I	0	8	**12.1** (9–14)	**1.0** (0.4–1.6)	**13.4** (11.4–14.9)	–
II	2	3	**21.5** (19–24)	**3.2** (2.9–4.0)		–
III	4	4	**28.0** (26–30)	**4.2** (2.0–7.2)		–
IV	8	3	**44.0** (24–57)	**6.4** (3.8–8.7)		–
V	16	4	**57.0** (37–85)	**6.1** (2.5–16.0)		+
VI	29	6	**234** (75–475)	**3.9** (0.7–10.0)	**12.4** (11.6–13.2)	++++
VII	38	4	**218** (127–370)	**4.5** (1.4–8.2)		+++
VIII	46	4	**87.3** (60–118)	**9.9** (4.5–16.5)	**12.7** (11.6–13.7)	++
IX	63	2	**68.5** (55–82)	**7.3** (3.5–11.5)	**11.1** (10.1–12.1)	++
X	90	4	**44.3** (18–56)	**9.1** (2.9–20.0)	**11.8** (11.2–12.6)	++‡

*Figures in bold-face type = average values; figures in parentheses = range of values. These are based on daily excretion from 6 or more rats during the same period, expressed in terms of excretion from control rats fed a basal diet.

†Expressed in optical-density units per cc. of serum.

‡Large tumors present in all rats.

though changes in liver morphology did not occur as a result of feeding the latter dye, the spleen was several times larger than normal and showed, histologically, an accumulation of red blood cells and pigment. In the rats fed 3′Me-DAB, excretion of fecal urobilin remained well above normal during the entire period studied, although there was a tendency for it to be slightly less elevated during the phase of bile-duct proliferation and bilirubin retention.

Blood-hemoglobin determinations were performed in order to determine whether anemia was associated with the hemolysis suspected to follow 3′Me-DAB administration (table 1). Hemoglobin concentrations were not altered significantly during the 90-day treatment, although the trend was in the direction of a slight anemia (table 1). Apparently the amount of hemolysis was not sufficient to overtax the regenerative systems of the body

Serum-bilirubin concentrations showed very marked elevation as a result of 3′Me-DAB administration. Diets containing 4-aminoazobenzene did not produce any significant increase in serum bilirubin. Whereas the over-all average for control rats fed the basal diet was approximately 11 optical-density units per ml., values in the range of 200 to 400 units per ml. were not uncommon following azo-dye treatment. By comparison, serum-bilirubin levels of 400 to 700 units per ml. were observed in 2 rats 4 to 6 days after the ligation of the common bile duct. From this data, it would seem that extensive bile stasis was occurring in the liver as a result of the dye treatment. The rate of the bilirubin coupling reaction indicated that the pigment, for the most part, reacted directly and probably resulted from regurgitation of bile from the bile capillaries.

Observations on concentration of serum bilirubin in relation to changes in liver histology were of great interest. In all 3 experiments the serum bile pigment generally took a gradual upward trend during the first 15 days, reaching levels 5 to 7 times normal. During this period, morphologic changes in the liver were, for the most part, absent. The increased serum pigment was due to the hemolysis described above or to parenchymal-cell damage. The highest values were observed during the 20- to 30-day period that followed. The maximum was reached at approximately 30 days in the first 2 experiments and at 21 days in the last experiment, depending, apparently, upon slight variations in experimental conditions. In all cases, values over 100 units were accompanied by active proliferation of bile-duct cells. In 4 of these rats, the extent of proliferation did not exceed 8 percent. However, strong mitotic activity was observed in the bile-duct epithelium. As bile-duct proliferation subsided and the livers took on a more cirrhotic appearance, grossly, serum bilirubin returned to more normal concentrations. It was interesting to note that the level was only slightly above normal in rats that showed extensive infiltration of liver tumors. It could only be concluded that serum bilirubin would be of little value in predicting the extent of tumor formation.

In general serum cholesterol tended to follow somewhat the same trend as serum bilirubin, but to a much lesser extent. In experiment 2 (table 2), the average value for serum cholesterol was significantly above that of the control animals after 22 days of 3'Me-DAB treatment. The highest values occurred at approximately the same time in experiment 3 (text-fig. 1), except that as a group these concentrations were not significantly different from normal. The higher values of serum cholesterol were also associated with hyperplasia of the bile-duct epithelium. Ligation of the common bile duct was found to increase serum-cholesterol levels 150 to 250 mg. per 100 ml.

TABLE 2.—*Serum-bilirubin and serum-cholesterol concentrations in rats ingesting 3'methyl-4-dimethylaminoazobenzene**

Group	Time (*days*)	No. of rats	Serum bilirubin†	Serum cholesterol (*mg./100 ml.*)	Bile-duct proliferation
I	0	5	**10** (7–14)	**66** (61–77)	—
II	6	5	**19** (10–26)	**57** (51–82)	—
III	14	4	**72** (32–178)	**78** (67–91)	—
IV	22	4	**115** (32–230)	**99** (87–120)	+
V	30	4	**211** (94–280)	**74** (59–91)	+++
VI	38	3	**173** (130–253)	**63** (62–64)	+++

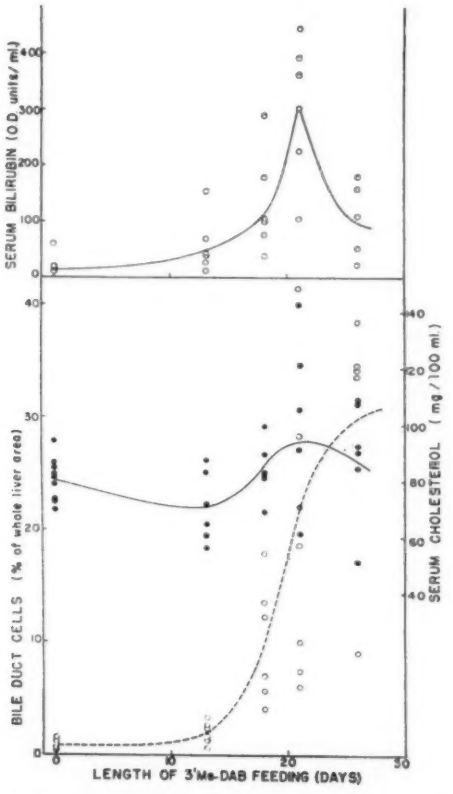
*Figures in bold-face type = average values; figures in parentheses = range of values.
†Expressed in optical-density units per cc. of serum.

Discussion

The destructive effect of azo dyes or their metabolites on red blood cells was described by Kinosita (*11*), who showed that abnormal cells were abundant after short periods of DAB treatment. Associated with the abnormal blood picture was a remarkably enlarged spleen in which stagnation of blood and destruction of red blood cells was a conspicuous feature. Edwards and White (*12*), also working with DAB, described an iron-containing pigment that deposited in both the liver and the spleen. These authors believed this pigment to be the result of increased red-blood-cell destruction, described above. Similar changes in both the liver and spleen were reported by Richardson and Borsos-Nachtnebel (*13*) to result from 3'Me-DAB administration. The data presented here on fecal-urobilin excretion provide further evidence that hemolysis is one of the consequences of azo-dye feeding. The reaction was not, however, found to be sufficiently extensive to produce significant anemia under the conditions employed. Since similar changes in urobilin excretion and in

spleen morphology were observed to follow treatment with the noncarcinogenic dye AAB, it does not appear that these alterations are involved in carcinogenesis *per se*.

With the information presented here it cannot be established whether intrahepatic bile-duct occlusion, which is indicated by the high serum bilirubin and cholesterol, precedes or is preceded by bile-duct proliferation.


SERUM BILIRUBIN (O.D. units/ml.)
BILE DUCT CELLS (% of whole liver area)
SERUM CHOLESTEROL (mg/100 ml.)
LENGTH OF 3'Me-DAB FEEDING (DAYS)

TEXT-FIGURE 1.—Variation of serum bilirubin and serum cholesterol during azo-dye feeding correlated with bile-duct cell proliferation.

●——● Serum-cholesterol level.
○——○ Serum-bilirubin level.
○·····○ Percentage of bile-duct cells in liver.

In all cases where high levels of bile constituents were observed, active proliferation of bile-duct epithelium was already in progress. This may indicate that the hyperplasia itself is responsible for occlusion of the bile passages, perhaps through poor structural arrangement of the cells. Histologically, duct lumina often seem to be absent during the first phases of proliferation. On the other hand, if it could be established that bile

stasis, with consequent high serum-bilirubin and cholesterol levels, actually preceded proliferation, the hypothesis suggested in the introduction would, to some extent, be substantiated. This hypothesis was based upon the fact that similar proliferations of bile-duct cells result from both ligation of the common bile duct and treatment with azo dyes. Serum bile constituents are elevated in both conditions, and it may be that the accumulation of some biliary substance in the tissue is responsible for the stimulus to the bile-duct cells. In the case of the azo dyes, this bile stasis may result from distention of hepatic cells in the peripheral zone, with consequent compression of bile capillaries. The inability of the cells in the central zone to store glycogen has been interpreted as stemming from a similar compression of sinusoids that results in centrolobular ischemia (*14*). The postulated cellular distention may arise as a result of a primary toxic effect of the dye similar to that which is termed "cloudy swelling." Further examination of the changes in bile-constituent levels in relation to changes in histology may provide a much needed physiologic explanation of the manner in which certain azo dyes are able to promote bile-duct proliferation and the subsequent morphologic changes of the liver.

Summary

1) The concentrations of serum bilirubin, serum cholesterol, fecal urobilin, and blood hemoglobin were examined in rats that had been fed the hepatic carcinogen 3′methyl-4-dimethylaminoazobenzene (3′Me-DAB) for varying periods of time. The results were compared with concurrent histologic changes in the liver.

2) Increased fecal-urobilin excretion, apparently arising from hemolysis, was found to result from the feeding of both 3′Me-DAB and the noncarcinogenic dye 4-aminoazobenzene.

3) Serum-bilirubin and serum-cholesterol levels were significantly above normal during the initial phases of bile-duct proliferation, indicating that this process is associated in some way with intrahepatic bile-duct occlusion. Although the gross appearance of cirrhosis and tumors characterize the later phases of the process, concurrent serum-bilirubin levels were only slightly above normal.

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Establishment of a Clone of Mouse Liver Cells from a Single Isolated Cell [1]

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In an earlier study, attempts were made to obtain a clone of mouse liver cells from a single cell (*1*). No isolated cell divided, and the concentration of chicken plasma required for the single-cell procedure was found to damage liver cells and prevent their survival.

The purpose of this study was to adapt mouse liver cells to grow in a plasma-clot substrate and to establish a clone of mouse liver cells from a single cell of such an adapted culture.

Materials and Methods

The cells used for adaptation and subsequent isolation originated from an explant of the liver of a 48-hour-old mouse of the Andervont line of strain C3H; the liver cells had been cultured under perforated cellophane for more than 3½ years (*2*).[3] During this interval and during the course of the present experiments, the fluid culture medium used was composed of 20 percent chick-embryo extract, 40 percent horse serum, and 40 percent Earle's balanced saline (*3*).

Although various methods of adapting the liver cells to continued growth in plasma clot were tried, only the following procedure was finally successful.

A dense cell suspension was prepared by scraping the cells from the cellophane substrate of a Carrel D-3.5 flask into the supernatant fluid medium by means of a platinum spatula (*4*). A volume of 0.4 ml. of this suspension was added to 0.2 ml. of chicken (hen) plasma in a Carrel D-3.5 flask, and a sheet of perforated cellophane (*5*) was lowered onto this soft clotting mixture.

After the cells had covered the substrate, the plasma clot, with the embedded cellophane sheet, was loosened, removed, and cut into explants approximately 2 × 8 × 25 mm. One ml. of chicken plasma was introduced into a Carrel D-3.5 flask; the flask was then gently agitated to

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] This strain, formerly designated 721, is currently designated NCTC strain 721, the prefixed letters being used to indicate all strains originating in the Tissue Culture Section, Laboratory of Biology, National Cancer Institute.

assure distribution of the plasma over the glass floor, and excess plasma was withdrawn to leave approximately 0.1 ml. adherent to the glass. To this was added 0.3 ml. of the fluid culture medium, and 1 explant was centered in each flask. After the soft clot had formed, 0.3 ml. of the fluid medium was added and the *p*H was adjusted by gassing with a mixture of 5 percent CO_2 in air. Fluid renewals were made thrice weekly.

When lysis of the plasma clot began, a new clot was formed by withdrawing the supernatant culture fluid and adding 0.2 ml. of chicken plasma to the culture flask containing the lysing clot.

In subsequent transplantations, the soft plasma-clot substrate that contained the cells was removed from the flask and was cut into explants approximately $2 \times 5 \times 12$ mm. Two or 3 explants were placed in each flask, to which 0.5 ml. of chicken plasma had been added. Clotting of the plasma was induced by adding 0.5 ml. of a suspension of liver cells that had been prepared from cells growing underneath the plasma clot on the glass floor of the flask. At subsequent fluid renewals those cultures with the harder plasma clots received amounts of culture fluid varying from 0.2 ml. to 1.5 ml., depending on the cell density.

Methods used for cell isolation and growth were essentially the same as those described previously (*6,1*). The suspension for isolation of single cells was prepared from the cells adhering to the floor of the flask after removal of the plasma clot. All embryo extract used in the fluid medium was filtered (*7*).

Results

Attempts were made to adapt cells, descended from 10 cellophane-substrate cultures, to grow in plasma. From 1 of these cultures, cells ultimately were successfully adapted to grow in hard plasma clot in the absence of cellophane and were serially subcultured for 18 transplant generations (11 months); a total of 195 cultures was prepared. Growth in a limited number of cultures was sufficiently rapid to require transplanting the cells at 7-day intervals; cells in the majority of cultures, however, grew considerably more slowly and, in a few instances, transplantation was not possible more often than every 43 days. In these plasma-substrate cultures, many cells remained on the glass floor of the flask after removal of the plasma clot; these remaining cells, suspended in fluid medium, were used for isolation of single cells or for inoculation of new cultures.

At the time of single-cell isolation, liver cells growing in plasma clot had been cultured for 10 transplant generations (6 months). In 2 experiments a total of 40 capillary segments was planted. Five single cells survived, and 3 of these divided; 2 divided to produce colonies of 3 to 10 cells and then died, and the third continued to proliferate to establish a clone culture. This culture was transplanted on the 77th day to a substrate of perforated cellophane embedded in a thin layer of plasma clot. Subsequent transplantations were made to cellophane substrate in Carrel D-3.5 flasks and ultimately to cellophane or to glass substrates in T-60 type culture vessels (*8*).

This clone of mouse liver cells, currently designated NCTC clone 1469, has now been maintained in culture for 5 years. Growth is luxuriant, and, after transplantation, cells cover the 60 cm.^{2} area floor of a T-60 flask as a dense sheet in 5 to 7 days.

No appreciable difference in culture design between cells of the parent strain and the derived clone could be recognized when the cells were grown either in plasma substrate or on the glass surface of the culture vessel (figs. 1–5).

Discussion

Previous studies had indicated that the plasma clot was the major factor interfering with proliferation of liver cells of this strain. In the present study, a substrate composed of very soft clot in combination with cellophane was used initially to adapt the liver cells to grow in the harder plasma clot. Use of progressively harder clots with elimination of cellophane allowed cells of this strain to adapt to plasma and ultimately to grow in a hard clot. The rate of growth, however, for the majority of adapted cultures appeared to be considerably slower than that of the cells grown on glass or cellophane substrates. Whether this adaptation of the cells to plasma involved a selection or cell transformation, or both, cannot be determined from the data available.

The behavior of this cell strain in requiring adaptation to a plasma substrate serves to emphasize the complexity of factors that may be involved in growth of single cells of certain types and successful establishment of clones therefrom.

Summary

A strain of mouse liver cells was adapted to grow in chicken plasma clot *in vitro*.

A clone of mouse liver cells was established from a single cell isolated from an adapted culture. This clone, designated NCTC clone 1469, has now been maintained in culture for 5 years.

Cells of both the parent strain and the derived clone show no appreciable differences in gross morphology or in general culture architecture.

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PLATE 51

FIGURE 1.—Liver cells of the original parent strain, NCTC strain 721, growing at the glass interface in hard plasma clot in a Carrel D-3.5 flask. This culture was prepared from a suspension of cells. *Note* variations both in complete cell separation and in cell size and shape. These cells were photographed 5 days after transfer to this flask and 83 days after first exposure to plasma. At this time the strain had been in culture for more than 8 years. × 200

FIGURE 2.—Liver cells of NCTC clone 1469 growing at the glass interface in hard plasma clot. This culture was prepared from a suspension of cells. *Note* similarities between these cells and cells of the original strain. These cells were photographed 5 days after transfer to this flask and 83 days after first exposure to plasma. The clone had been maintained in culture for 4 years at this time. The photograph was made the same day and under conditions comparable to those for figure 1. × 200

FIGURE 3.—Cells of the original liver strain, NCTC strain 721, growing on glass substrate in a Carrel D-3.5 flask. These cells were photographed 4 days after transfer to this flask, on the same day and under conditions comparable to those for the cells in figure 1. At this time the strain had been in culture for more than 8 years. × 200

FIGURE 4.—Liver cells of NCTC clone 1469 growing on glass substrate in a Carrel D-3.5 flask. *Note* similarities between cells in figures 3 and 4. These cells were photographed 4 days after transfer to this flask. The clone had been maintained in culture for 4 years at this time. × 200

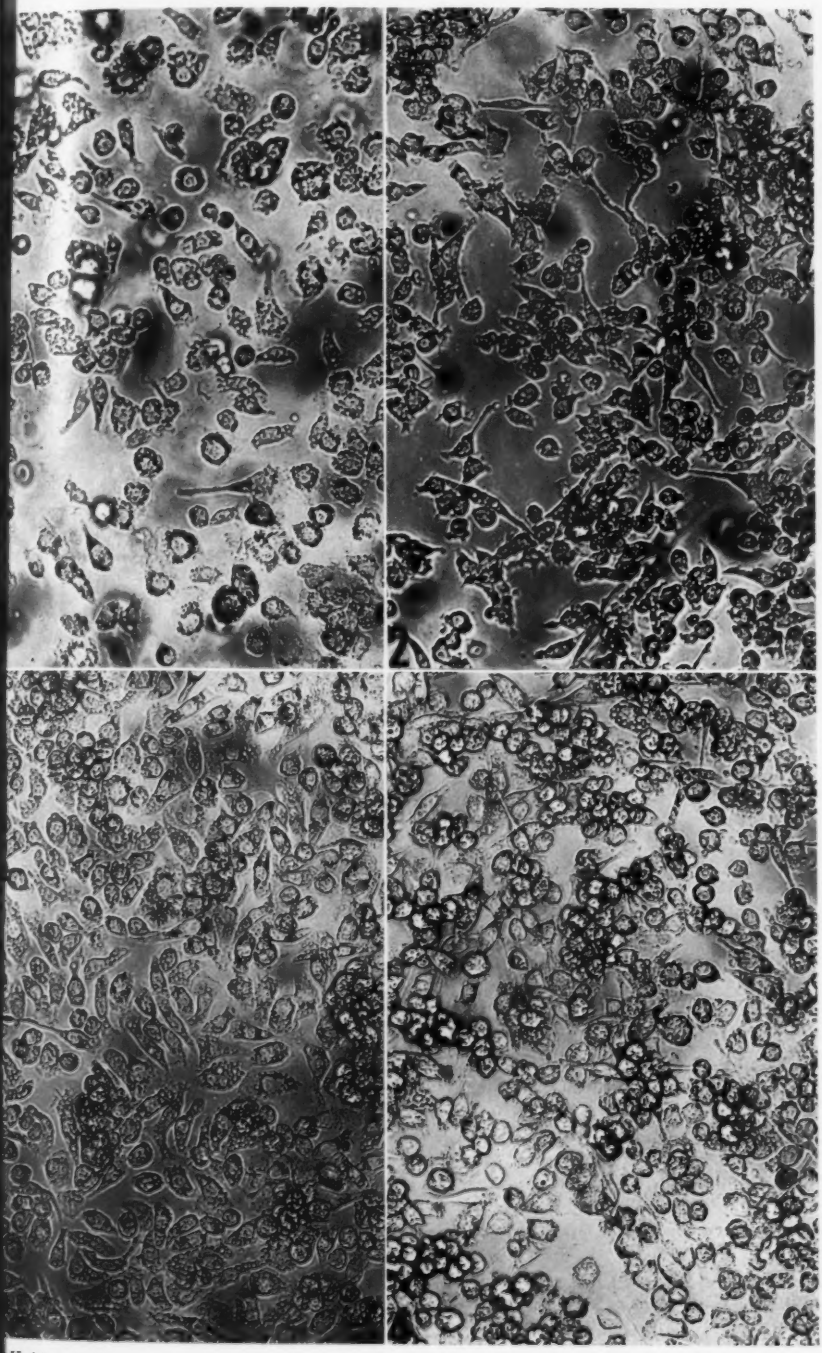

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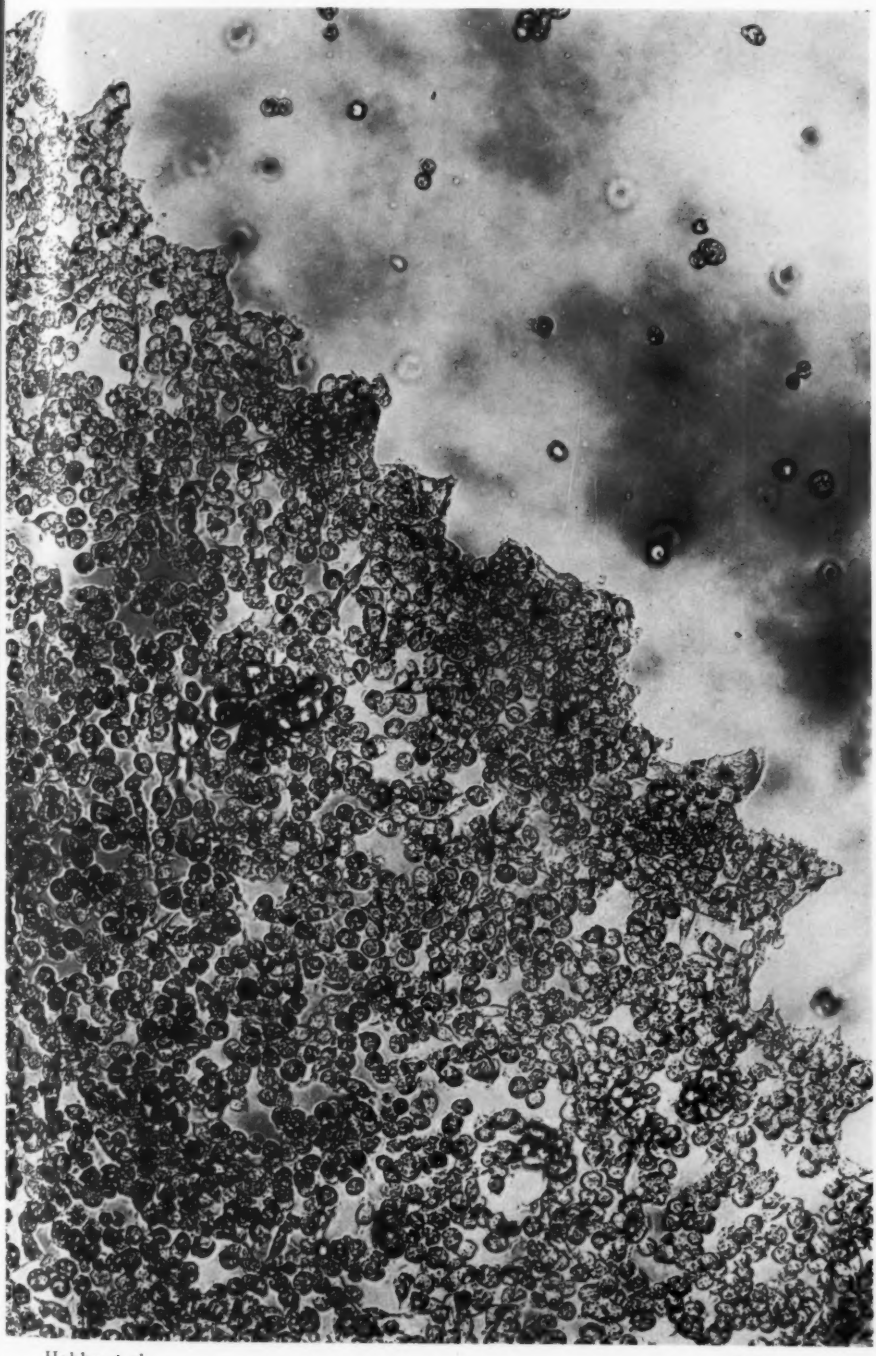
PLATE 52

FIGURE 5.—Liver cells of NCTC clone 1469 growing from an explant in hard plasma clot. *Note* packing of the cells at the margin of the culture, somewhat simulating an epithelium-like growth pattern. These cells were photographed 11 days after transfer to this flask and 72 days after initial exposure to plasma. The clone had been maintained in culture for 4 years at this time. × 200



Establishment of Clones of Epithelial Cells from Human Skin [1, 2]

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The Tissue Bank, Naval Medical School, and the Tissue Culture Section, Laboratory of Biology, National Cancer Institute, have collaborated in a project to grow cells from human skin. As a result of this project, a strain of epithelial cells isolated from the normal skin of a 65-year-old man has been maintained in culture for 3 years. The earlier history of this cell strain, designated NCTC strain 1769,[5] has already been reported (*1*).

If successful therapy is to be given to the patient who is critically burned or whose skin is otherwise denuded, a better understanding of the behavior of cells of this strain is clearly necessary. This paper describes the successful establishment of cloned strains of these cells and describes the *in vitro* behavior to the present time of cells of the clones.

Materials and Methods

Cell maintenance.—Since its original establishment in November, 1953, NCTC strain 1769 has been cultivated on 30 percent pooled human serum, 20 percent hyaluronidase-treated chick-embryo extract, and 50 percent Earle's balanced saline (*2*). In general, the handling of this strain is comparable to that of strain NCTC clone L-929, except that human serum has been used. One culture, maintained in a T-60 flask on the human stock medium, permits weekly explantation of 10 subcultures. In these, proliferation is so rapid that within a few days the cells have covered the floor of each flask with a sheet of closely adherent cells.

Preparation of culture media.—In the first attempt to obtain a clone of the skin epithelium (experiment #2271) plasma was prepared by collecting blood from adult hens in precooled, paraffin-lined bottles. The blood was

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[2] The conclusions and opinions expressed in this paper are those of the authors and are not to be construed as official or necessarily reflecting those of the Medical Department of the U. S. Navy or of the Naval Service at large.

[3] Tissue Bank, Naval Medical School, National Naval Medical Center.

[4] Tissue Culture Section, Laboratory of Biology, National Cancer Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[5] Since references to specific cell strains are becoming increasingly frequent in the tissue-culture literature, in order to avoid confusion in the future, all strains originating in the Tissue Culture Laboratory, National Cancer Institute, will be designated "NCTC strain," followed by the strain number. Cloned strains will be designated "NCTC clone," followed by the strain number.

centrifuged at 2300 r.p.m. (1600 × g) for 20 minutes and the supernatant plasma was transferred to fresh paraffin-lined bottles for storage until use. Immediately before use, the plasma was recentrifuged for 5 minutes. When this unfiltered, although centrifuged, chicken plasma was used, the plasma clots in some of the cultures were contaminated with what appeared to be chicken leukocytes. Therefore, in attempts to establish the second clone (NCTC clone 2414), all chicken plasma was prepared by filtration under pressure through a #03 Selas filter,[6] as described by Bryant *et al.* (*3*). All other media used in both experiments were sterilized similarly.

Source of cells for single-cell isolations.—All cultures of the parent NCTC strain 1769 used in this study were grown in Carrel D-3.5 flasks in culture media composed of 30 percent pooled human serum, 20 percent hyaluronidase-treated chick-embryo extract, and 50 percent Earle's balanced saline (*3*). This medium will hereafter be referred to as human stock medium. No plasma was used; the glass floor of the flask was the only substrate.

Procedure for single-cell isolation and growth.—A cell suspension was prepared by shaking the cells of 24-hour-old cultures free from the glass surface of the flasks. The suspension was then passed through a platinum-alloy double sieve of 80 and 150 mesh (*4*). Capillary pipettes were lined with a thin coating of chicken plasma and prepared according to the procedures described by Sanford *et al.* (*5*) and Likely *et al.* (*6*). In experiment #2271, the plasma was unfiltered; filtered plasma was used in experiment #2414.

After incubation for 18 hours the sealed capillaries were examined under a microscope. Capillaries with suitable longitudinal distributions of cells were severed into lengths measuring approximately 5 mm. with a Bard-Parker #11 knife blade mounted on a #7 handle. Each segment was placed in a separate Carrel D-3.5 flask containing 0.5 ml. chicken plasma mixed with 0.5 ml. human stock medium. The medium containing the capillary segments was allowed to clot, and then an additional 1 ml. of human stock medium was added to each flask. The flasks were then gassed with humidified 5 percent CO_2 in air, stoppered, labeled, and incubated at 37.5° C.

Results

The results of attempts to establish the 2 clones of human skin epithelium are shown in table 1.

After 36 hours of incubation, all cultures containing more than 1 cell were discarded; 2 cultures were carried in experiment #2271 and 1 in experiment #2414.

At the end of 10 days, patchy areas of cells, which were assumed to be a contamination from the unfiltered plasma, appeared in the plasma clot of both cultures in experiment #2271 (fig. 1). No attempt was made to disturb the capillaries until the 14th day, at which time they were transferred. The capillary in the first contaminated flask was trans-

[6] Selas Filter Corporation, Philadelphia, Penna.

TABLE 1.—*Data on single-cell experiment*

	Experiment #2271	Experiment #2414
No. capillary preparations	30	19
No. capillary segments planted	62	27
No. single-cell preparations after 18 hours	6	5
Total no. single cells undergoing mitosis	4	3
No. single cells dividing to produce 10 or more cells	2	1

ferred by lifting it free of its plasma milieu and placing it in a fresh flask containing filtered plasma; the cell outgrowth from the tip of the capillary was left behind and discarded. The capillary in the second contaminated flask was transferred in a similar manner, except that a small surrounding area of cell-free clot that had been left attached to the capillary was transferred with it. To each flask, 0.5 ml. of the human stock medium was added to form a clot. An additional 1.0 ml. of human stock medium was added as a supernatant layer.

Five days after transfer a luxurious outgrowth of cells from the capillary in the first flask was observed, as shown in figure 4. Critical microscopic examination showed that there were no contaminating chicken cells in the surrounding medium. However, contaminating cells were observed in the second flask, and it was discarded. The outgrowth in the first flask continued rapidly until the 23rd day after transfer, at which time a single third-generation subculture was made in a T-30 flask. This was designated NCTC clone 2271.

In experiment #2414, in which only filtered medium was used, the isolated cells in 3 capillaries showed mitosis; 1 of the cultures continued to proliferate rapidly and was designated NCTC clone 2414.

Cultures of both NCTC clone 2271 and NCTC clone 2414 were later transferred to and maintained in T-60 flasks; the culture fluid was changed routinely 3 times a week. When a cell suspension of either clone was transferred, a dense sheet of cells generally covered the floor of the flask in about 5 to 7 days. Transfer of these clones is now made about 5 to 7 days after explantation.

The cell outgrowth of each epithelial clone from the open ends of the capillary was characterized by both terminal and lateral cohesion of the sheet-like cells (fig. 5). This culture architecture contrasts strongly with that of the fibroblast-like cells of NCTC clone L-929 at a comparable stage (fig. 4).

Cells of the epithelial clones were easily separated from the glass floor of the flask by vigorous shaking. The resulting cell suspension was then transplanted by use of a pipette or special burette. When planted, the cells soon adhered to the glass floor of the flask and often tended to grow so that each cell or cell clump gave rise to a small island from whose peripheral zones free elongate cells migrated. These eventually coalesced

into larger areas of cells, which in turn had a definite tendency to coalesce with other nearby cell areas to form more or less continuous sheets. Still later, as the population density increased further, each cell was appressed to those around it (fig. 3) and tended to become polygonal or spherical. In other instances, where a new culture was seeded with a more uniform suspension of cells, the cells often tended to form a loose, netlike architecture on the glass surface, which gradually became increasingly dense until ultimately a large, closely packed sheet of cells was produced.

Discussion

The original work of Sanford *et al.* (*5*) on the growth *in vitro* of single cells was done with the mouse fibroblast. At that time, they raised the question whether this procedure could be used with other cell types. Subsequently, Likely *et al.* (*6*) showed that clones could be established from single cells of both chick and mouse origin. Our success in establishing a clone of epithelium from the normal skin of a 65-year-old man is a practical extension of this method to still another type of cell.

In our experience with thousands of cultures in the Tissue Culture Laboratory, the present instance (expt. #2271) is the only one in which extraneous cells were found in chicken plasma. However, microscopic examination showed that those few cells that appeared in the clot area soon died and disintegrated. In all clone cultures, from the first one grown successfully, filtration of the serum, embryo extract, and saline has precluded possible contamination from these sources. In the previously established clones, rigorous and critical initial microscopic examination of the cultures, both at the 1-cell stage and in later development, showed no evidence of extraneous cells from the plasma. There is no doubt, however, that use of filtered plasma, as in experiment #2414, does facilitate preparation of single-cell cultures by reducing the amount of time spent in microscopic examination. For this reason, use of filtered plasma, along with other filtered solutions, is now routine in this laboratory.

Using chicken and mouse cells, Likely *et al.* (*6*) reported that 17 percent of the capillary segments contained living cells 18 hours after planting, and of these single cells, 2.7 percent multiplied to 10 or more cells. In the present study with human skin epithelium, as shown in table 1, 23 percent of the capillary segments yielded single cells 18 hours after explanation and, of these, 26 percent multiplied to 10 or more cells. This may indicate that, in general, cloning a pure strain of cells is easier with human tissue than with other cell types. However, to a limited extent at least, the greater degree of success certainly arises from increased facility with the method.

It has been demonstrated by Puck *et al.* (*7*) that the plating efficiency of his method with cells of the HeLa strain of human epidermoid carcinoma is about 50 to 70 percent and that when maximal care is taken with trypsinization, it approaches 100 percent. At the present time, in our view each of the 2 methods of cloning has its own specific area of use-

fulness, but the data available do not yet allow a critical comparative evaluation.

While the general morphology and mode of growth of NCTC clone 2414 strongly contrast with those of NCTC clone L-929, criteria which will conclusively distinguish this epithelial-cell type from all other cell types have not yet been established. Characterization must, of necessity, evolve from studies that include comparisons of internal cell structure, cell behavior under defined experimental conditions, and cell metabolism and nutrition.

Summary

Two clones of epithelial cells from the normal skin of a 65-year-old man have been established. As cells of both clones migrated from the open ends of the capillaries in which they were grown, they tended to cohere laterally and terminally to form a continuous sheet. This general pattern of growth contrasts strongly with the loose or terminally coherent structure formed by clones of fibroblast-like cells.

The cells of NCTC clone 2271 were discarded recently after continued rapid proliferation *in vitro* for over 10 months. NCTC clone 2414 has been carried for more than 11 months and will be used in further experimental work.

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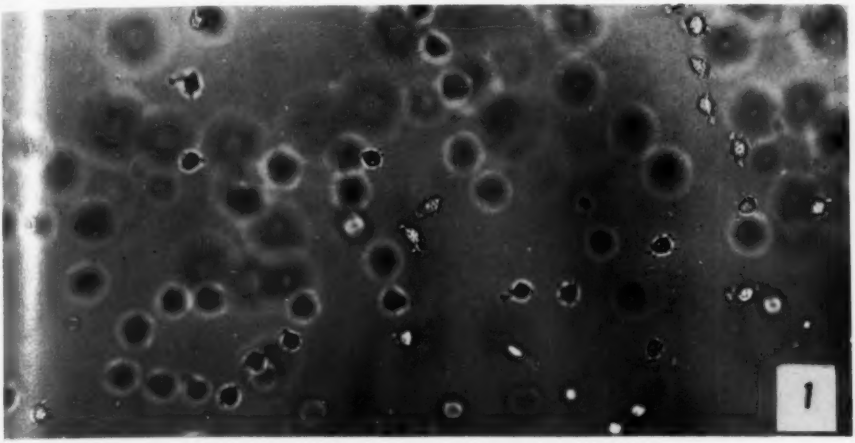
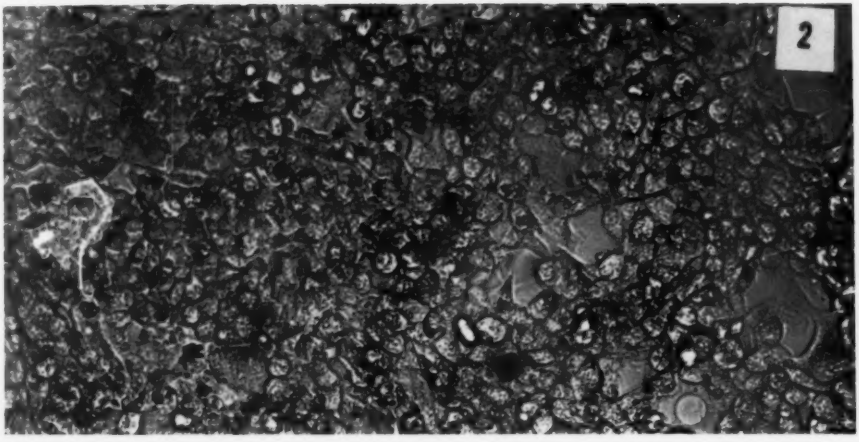
PLATE 53

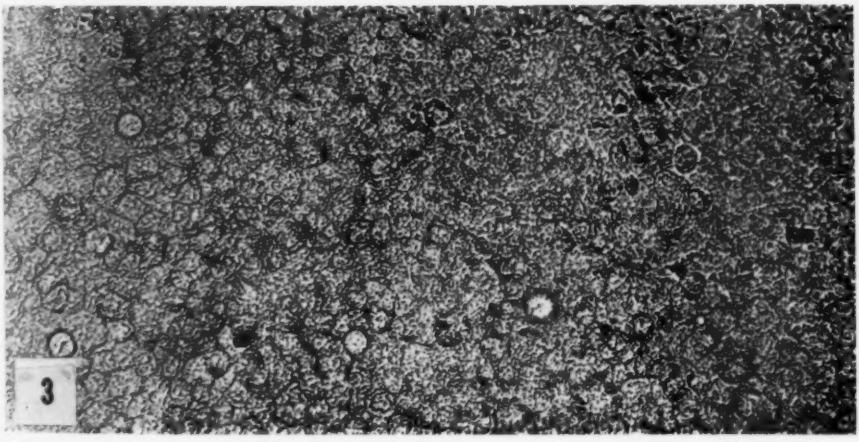
FIGURE 1.—Chicken cells as observed in the plasma clot of a culture in experiment #2271. Some time later these cells disintegrated. × 200

FIGURE 2.—Photograph of cells of NCTC strain 1769 in human stock medium 682 days after explantation and 3 days after transfer to a D-3.5 flask. Rapid cell proliferation has already resulted in the production of a coherent cell sheet. × 200

FIGURE 3.—Photograph of NCTC clone 2414 48 days after isolation as a clone and 3 days after transfer to D-3.5 flask. Rapid cell proliferation has resulted in a coherent cell sheet. The greater crowding of these cells, as compared with those of figure 2, is due to greater population density, probably from a heavier initial population in the suspension used to plant the culture. × 200







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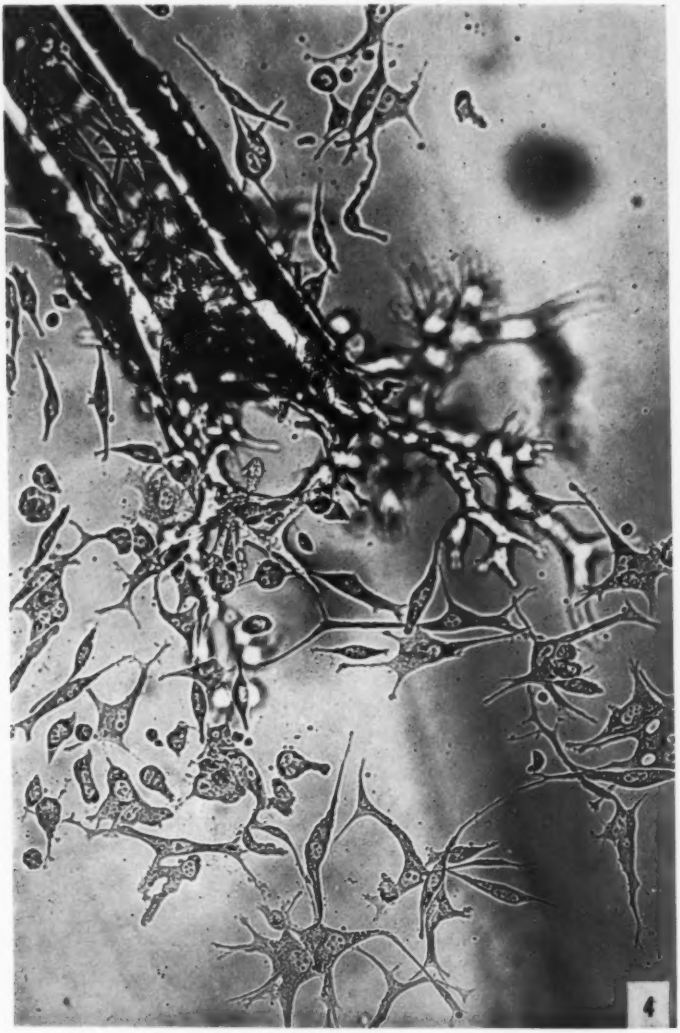

Perry *et al.*

PLATE 54

FIGURE 4.—NCTC clone L-929 mouse fibroblast cells migrating from a single-cell capillary. Elongation and terminal cohesion are predominant. Compare with cell morphology shown in figure 5. This photograph same as figure 8, page 237, *Journal of the National Cancer Institute*, volume 9, 1948.

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PLATE 55

FIGURE 5.—Capillary fragment, transferred free of contaminating chicken cells, in a D-3.5 flask for 5 days with filtered chicken plasma. *Note* the confluent outgrowth of cells as compared to the outgrowth in figure 4. At a comparable stage NCTC clone 2414 was similar in architecture. × 200

A Description of Spontaneous Congenital Testicular Teratomas in Strain 129 Mice[1,2]

L. C. STEVENS *and* K. P. HUMMEL, *Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine*

A rare mouse tumor, teratoma testis, develops spontaneously in about 1 percent of inbred strain 129 male mice (*1*). In addition to their inherited susceptibility to this type of tumor, there are other advantages of using strain 129 mice for the study of teratomas: They are available in large numbers; they are genetically homogeneous except for a single coat-color gene (chinchilla or albino); and the size of the mouse testis makes it practicable to study serial sections of the entire gonad.

The structure and tissue interrelationships of testicular teratomas in mammals have been studied most thoroughly in humans and horses. Willis (*2*) studied microscopic sections from serial slabs of 14 (6 testicular) human teratomas and mapped the identifiable tissues to show their distribution and relationships. He observed a wide variety of immature and adult tissues, including many kinds of epitheliums in cysts and glandular structures. These ranged from poorly differentiated components resembling a variety of types of "adenocarcinoma" to organoid formations. Immature neuroepithelium and neuroglial tissue occurred but apparently no nerve cells were observed. Cartilage—sometimes showing ossification—skeletal and smooth muscle, and fat were also present. Other investigators have described a similar array of tissues in human teratomas (*3–6, et al.*). Most human testicular teratomas consist primarily of immature tissues and are malignant.

The 3 (1 bilateral) cases of equine teratoma testis described by Willis and Willis and Rudduck (*7,8*) resembled the human tumors in the types of adult tissues found. The most conspicuous difference was that the tissues in the teratomas of horses were differentiated, and the tumors were designated as benign growths.

Although Willis found tissue correlations, such as glandular epitheliums with smooth muscle and immature nervous tissue with cartilage, he stated, "Most teratomas exhibit no sign of somatic axiation, segmentation, or delamination of germ layers; they possess no organs or true somatic regions; they exhibit anomalous excess of some components, and anoma-

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[2] This investigation was supported by research grant C-2662 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and by a grant from the American Cancer Society, Inc.

lous absence of others; and they exhibit abnormal mixtures and relationships of tissues and co-existence of tissues of widely different degrees of maturity." He concluded that a teratoma is not homologous with a fetus and rejected the twin-inclusion and parthenogenetic hypotheses.

Teratoid tumors of the human testis have been classified into 2 subtypes (*4,9,10*). One subtype, "teratocarcinoma," includes those in which differentiation has occurred in some areas and tissues of the tumor, while other parts of the tumor are relatively undifferentiated. The second subtype, "teratoma," occurs rarely and includes completely differentiated adult-type tissues. According to this system of classification, strain 129 mice develop both subtypes. However, we prefer to regard all of our tumors as representing a single condition and have adopted the nomenclature proposed by Willis (*11*), who designates this disease as teratoma.

The study reported here was undertaken in an effort to provide a basis for the comparison of testicular teratomas occurring in strain 129 mice with those found in the testes of humans and horses. For the sake of uniformity, the main body of this paper will deal with teratomas found in mice 20 to 30 days of age. Features of teratomas of older mice that differentiate them from teratomas of mice of weaning age will be mentioned. A study of teratomas found in the testes of mice ranging in age from newborn to 20 days is currently in progress.

Materials

The teratomas to be described were derived from several colonies of inbred strain 129 mice maintained at this laboratory. The mice were fed Purina laboratory chow and provided with water *ad libitum*. Neither the mice examined nor their parents had received treatment to which the relatively high incidence of teratomas might be attributed.

The distribution within age groups of the mice which have been studied is as follows:

Age of mice with teratoma	No. of teratomas examined
0–20 days	32
20–30 days	79
31 days–3 months	19
over 3 months	17

Most of the tumorous testes were placed *in toto* in vials containing Vandegrift's fixative, serially sectioned, and stained with hematoxylin and eosin.

Observations

Gross

The testicular teratomas of strain 129 mice are easily detected during *post-mortem* examinations of animals 1 week of age or older. The tumors enlarge the testes, and hemorrhagic areas are characteristic. In the 20-to-30-day-old group, the presence of a tumor may increase the diameter of the testis along its longitudinal axis from approximately 5 mm. to 20 mm. The tumor frequently deforms the testis, but the capsule is never

broken. The anterior, posterior, or midportion of the testis may be involved but not the epididymis. In testicular tumors, one can identify grossly such differentiated tissues as bone, red marrow, cartilage, fat, epithelial cysts, and squamous epithelial pearls interspersed among grayish to white masses. Occasionally, pulsating or twitching muscle movements are seen. The muscle may continue to twitch after the testis is removed from the mouse. In a few cases, muscle beating rhythmically, at the rate of about 30 times a minute, was observed in teratomas for several minutes after the animals had been sacrificed. Histologically these tumors revealed an abundance of heart muscle.

Most of the tumors in old mice were much enlarged, mainly by cysts filled with fluid and stagnant blood. It was previously reported that about 75 percent of the tumors were located in the left testis (*1*). Data collected more recently confirms this—the left side being affected in 124 of 166 cases. Only 2 cases of bilateral teratomas were encountered.

Approximately 5 percent of strain 129 males show another congenital testicular abnormality—small testis, which also occurs primarily on the left side. In newborns this condition is manifested by extensive hemorrhage into the testis and degeneration of some or all of the spermatogenic tubules.

Other abnormalities of the reproductive system are rare in strain 129 males. Of the many thousands of males autopsied, a few cases of cryptorchidism, hermaphroditism, and failure of the scrotum to develop have been observed, but the incidence of these anomalies is far less than the incidence of testicular teratomas. No teratomas have been observed in the thousands of strain 129 females autopsied at all ages. It has been mentioned that in 1 case an ovarian teratoma developed in a (129 $\times$ DBA)F_1 hybrid (*1*).

The testes of adult mice are readily retractable from the scrotum to an abdominal position. When the testis is markedly enlarged by a teratoma, it is unable to traverse the inguinal canal and becomes locked in the scrotum or in the abdomen. None of the tumorous testes were cryptorchidic for any other reasons.

Microscopic

The tumors in mice 20 to 30 days of age vary in size from about one fourth to several times the testicular volume. Nearly all have conspicuous hemorrhages, usually surrounded by neural tissue. The tumors are not encapsulated and the teratomatous elements are interspersed among the spermatogenic tubules. Well-formed arteries and veins course through them.

In several cases where the tumor was relatively small, it was possible to determine that the rete was clearly separated from the tumor by spermatogenic tubules and was not involved in neoplastic change.

In general, identification of the tissues in these teratomas is relatively easy. Usually the cells of the various tissue types are grouped in more or less well-defined areas large enough to permit recognition. The general

appearance bears a close resemblance to the tissuelike groupings in reaggregates of dissociated chick limb-bud cells cultivated *in vitro* by Moscona (*12*). The difficulties mentioned by Willis (*2*) in identifying embryonic and immature tissues in human testicular teratomas were encountered here to a lesser degree, since most of the tumors are composed of well-differentiated tissues with their immature stages adjacent to them. The frequency with which the various tissues occur in the teratomas varies from 100 percent for nervous tissue to about 7 percent for hair follicles and sebaceous glands. Table 1 shows the frequency of various tissue types found in 67 tumors of 20-to-30-day-old mice. Not infrequently, the spermatogenic tubules in the tumorous areas are ruptured and free spermatocytes are scattered among teratomatous tissues.

Approximately two thirds of the teratomas were composed of fully differentiated tissues in which no mitotic activity was observed. The rest had dividing embryonic cells.

Multiple teratomas were not observed in any of the testes of 20-to-30-day-old mice. However, examination of tumors in young mice indicated that 2 or more independent adjacent foci may grow together to form a single tumor mass. In 1 teratoma in a 3-day-old mouse, 2 distinct foci were separated by testicular tissue. In another testis of this age, there were 3 apparently independent foci with contiguous borders.

TABLE 1.—*Incidence of tissues types in 67 testicular teratomas in mice 20 to 30 days of age*

Tissue types	Percentage
Nervous tissue	
Neuroglia	100
Neurons	100
Motor neurons	61
Immature neuroepithelium	31
Ganglion cells	16
Epitheliums	
Cuboidal nonciliated	97
Cuboidal ciliated	97
Simple squamous	95
Goblet cells	93
Columnar ciliated	92
Pseudostratified ciliated	81
Stratified squamous	69
Pseudostratified nonciliated	65
Tubuloalveolar glandular	60
Columnar	52
Transitional	48
Epidermal (with hair and sebaceous glands)	7
Connective tissues	
Areolar and dense fibrous connective tissue	100
Cartilage	84
Bone	83
Bone with marrow	69
Adipose	21
Muscle	
Smooth	37
Striated skeletal	36
Cardiac	30
Others	
Notochord or adrenal medulla (not positively identified)	97
Embryonic mesenchyme	25

The component tissues of the teratomas found in mice 20 to 30 days of age will be described in the order of the frequency of occurrence.

Nervous tissue.—Nervous tissue, present in all of the teratomas examined, is represented by immature neuroepithelium lining canals, gray matter supported by neuroglial cells, and ganglia.

About a third of the tumors have areas of immature neuroepithelium arranged as distorted tubules (fig. 1) or in rosettes. The ependymal layer shows many mitotic figures and appears to give rise to a dense mantle layer surrounding it. The mantle layer may be surrounded by a marginal layer and an external limiting membrane or may spread out into differentiated neural tissue. Some of the cells of the mantle layer contain mitotic figures.

Mature neuroepithelium lining canals is commonly seen. The canals are often filled with blood that may exude into the intercellular spaces of the surrounding neural tissue.

Neurons supported by neuroglial cells are seen more frequently and in greater abundance than other cell types. Over half the teratomas have neurons with large cell bodies of the type found in the ventral horn of the spinal cord (fig. 2). Occasionally, gray matter is enclosed and subdivided into lobules by meninges-like sheaths of connective tissue with blood vessels. More often, the neural tissue is not confined by connective tissue but forms a broad field containing areas of other tissues.

Ganglia of various sizes, composed of nerve cells surrounded by sheath cells, occur in about 16 percent of the tumors examined (fig. 3). Usually fascicles of nerve fibers pass from these structures into adjoining areas, where they disappear. No nerve endings on muscle fibers or other structures were observed.

Epitheliums.—Several types of epithelium line cysts and tubules and form acini and other glandular structures. Cysts are commonly lined with epitheliums of different types that are contiguous with one another. An epithelial sheet may have, for example, stratified squamous epithelium abutting columnar epithelium with goblet cells (fig. 4). Willis (*2*) noted direct continuity of differentiating central nervous tissue with young squamous epithelium. We have not observed neuroepithelium continuous with non-neural epithelium.

The simple epitheliums include squamous, cuboidal, columnar, and pseudostratified types. These may be ciliated and may have goblet cells interspersed. Sheets of mucus-secreting cells line cysts and form acini. Glandular structures, composed of mucus- or serum-secreting cells, or a mixture of the 2, and resembling pancreas or salivary glands, may drain into cysts by way of ducts (figs. 5, 6). Cysts may be distended by the accumulation of fluid secreted by the cells lining the cysts and the connecting glands. Pseudostratified columnar ciliated epithelium, respiratory in type, is found characteristically. Usually goblet cells are present. Transitional epithelium of the type found in the urinary tract is common, but renal tissue has not been recognized.

Cysts lined by stratified squamous epithelium and containing exfoliated

keratinized cells occur frequently (figs. 7, 8). Occasionally, cysts of this type are associated with hair follicles and sebaceous glands, which indicate their epidermal character (figs. 7, 8). More often, the stratified squamous epithelium is like that found in the oropharynx and esophagus.

Connective tissues.—Embryonic mesenchyme (fig. 9) occurs in about a quarter of the tumors 20 to 30 days of age. Dense fibrous and loose areolar connective tissues are found in all the tumors examined.

Lobules of brown fetal fat, adult adipose tissue, and transitional stages occur in about one fifth of the teratomas. Occasionally, patches of fat cells are present in the bone-marrow spaces.

Compact clusters of precartilaginous mesenchyme and nodules of definitive hyaline cartilage are common. Mitoses may be seen in the cartilage cells and in the surrounding perichondrium. Both intramembranous and endochondral bone occur, and in each the presence of numerous osteoblasts is evidence of active bone formation. Endochondral ossification resembles that occurring at the epiphyses of long bones (fig. 10). Here cartilage cells arranged in columns may be seen undergoing degeneration, which leaves a matrix framework upon which spongy bone is being laid down. Compact bone occurs adjacent to the periosteum.

In the cavities of the spongy bone, myeloid tissue composed of cells of varied size and staining capacity, characteristic of red marrow, can be seen (fig. 10). Megakaryocytes, reticulum cells, and mature and immature blood cells, as well as prominent blood sinuses, attest to active hematopoiesis in these areas.

Muscle.—Skeletal, cardiac, and smooth muscle are present in about one third of the teratomas (figs. 11, 12, 13). The skeletal muscle is attached directly to perichondrium or periosteum or may be inserted by way of tendons. Dividing immature muscle fibers may be seen near spindle-shaped cells, which are probably myoblastic in nature.

In some cases it is difficult to distinguish between cardiac and skeletal muscle. Frequently, muscle bands are not oriented in a way that makes clear such characteristics as branching, nuclear position, or intercalated discs.

Smooth muscle occurs encircling epithelium-lined cavities, which gives them the appearance of respiratory or alimentary structures.

Endocrine tissues.—In a few of the teratomas there are nodules that in cellular arrangement resemble endocrine glands (fig. 14), such as adrenal cortex, anterior pituitary, or islets of Langerhans. Although it is impossible to establish their identity, they lack ducts and have the blood-vessel networks characteristic of an endocrine tissue. In one case a nodule had the appearance of thyroid tissue, but again positive identification is not claimed.

Ninety-seven percent of all tumors contain clusters of cells that we have not been able to identify. These cells have granular nuclei, foamy cytoplasm, and distinct cell membranes (fig. 15). Occasional mitoses are seen. Each cluster is enclosed by a membrane, and several may occur adjacent to one another. Frequently, spicules of bone are found within

or adjacent to them (fig. 16). They are found in transplantable testicular teratomas of strain 129 mice, which indicates that they are a component of the tumor. This tissue bears a strong resemblance to fetal notochord and also resembles adrenal medulla.

Testicular Teratomas in Mice Older than 30 Days

In general, the teratomas found in animals 1 year of age and older resemble those in the 20-to-30-day age group with respect to the kinds of tissues represented. Although quantitative studies were not made, it is apparent that there is proportionately less nervous tissue and more epithelial tissue than in teratomas of younger animals (figs. 17, 18). Dense fibrous connective tissue is much more predominant in the tumors of older animals. Cartilage is more completely ossified, and immature and embryonic tissues are rarely found. The tumors in animals from 30 days of age to a year show stages of maturation intermediate to those described for the young and old-age groups.

Tissue Interrelationships

In the more differentiated teratomas, certain tissue interrelationships clearly exist. Columnar or pseudostratified epithelium is frequently encountered, together with satellite glands and a connective-tissue submucosa surrounded by smooth muscle (fig. 17). The muscle may be in 2 layers whose fibers are oriented perpendicular to one another. The mucosa may be thrown into regular folds resembling intestinal villi (fig. 18). These tissue associations give the appearance of parts of the respiratory or alimentary systems. No association appears to exist between tracheal epithelium and rings of cartilage, as described in many human teratomas.

Skeletal muscle is frequently found attached to bone or cartilage (fig. 14). When fat is present, it is commonly located around skeletal muscle, clusters of ganglion cells, epidermal cysts, and in bone-marrow cavities. Hair follicles and sebaceous glands are always found in association with keratinized stratified squamous epithelium, with the hairs projecting into the lumen of the cyst.

Tissues Not Encountered in Strain 129 Teratomas

Although many kinds of tissue are regularly found in these teratomas, it may be important to point out some that are absent. Pigment cells, a conspicuous component of mouse ovarian teratomas, have not been seen in these tumors. Since this could be attributed to the fact that strain 129 mice are albinos or contain pigment much diluted by the genes chinchilla (c^{ch}) and pink eye (p), strain 129 mice were crossed with DBA mice in order to introduce the genes C and P, yielding full black-agouti coat color. These mice have been backcrossed for 10 generations to strain 129 mice. Six teratomas have been found in fully pigmented mice derived from these crosses, but none has contained pigment cells. This

seems surprising in view of the presence of other neural-crest derivatives, such as spinal ganglia, sheath cells, and possibly adrenal medulla. Perhaps melanocytes will be found in these tumors in the future.

In his discussion of testicular tumors, Lewis (*5*) mentions the formation of blastocysts with differentiated cytotrophoblast or syncytiotrophoblast in a considerable percentage of teratoid tumors. We have been unable to identify these elements in our material. Other types of testicular neoplasms, such as seminoma, chorio-epithelioma, and interstitial-cell tumors, have not been observed.

Teeth and hepatic, renal, gonadal, and lung tissues are found only occasionally or not at all in human or equine testicular teratomas and have not been recognized in the neoplasms described here.

Transplantable Strain 129 Teratomas

Usually, even tumors that histologically showed dividing embryonic cells ceased proliferation after they were transplanted subcutaneously into other hosts. However, 4 of 19 strain 129 teratomas grafted subcutaneously have been shown to be capable of indefinite progressive gro wth as transplantable tumors. One of these tumors, in the 65th tran splant generation, is composed of undifferentiated cells. Another, after about 40 generations, still produces, in addition to undifferentiated cells, nervous tissue, cartilage, bone, various types of epithelial cysts, glandular structures, muscle, and fat. Descriptions of the growth and differentiation patterns of sublines of transplantable testicular teratomas will be reported elsewhere. The failure of strain 129 teratomas to metastasize is not surprising, since mouse tumors do so infrequently. However, one might consider that progressive growth of tumor grafts to hosts of the same strain represents "artificial metastases."

Discussion

Judging from the descriptions of human and equine testicular teratomas the congenital teratomas that develop spontaneously in the testes of strain 129 mice resemble them in many respects. In both human and equine teratomas, a wide variety of embryonic and adult tissues occurs without organized arrangement.

Neural tissue is present in all strain 129 teratomas observed in animals 20 days of age and older. Willis (*11*) stated that nervous tissue is present in at least 80 percent of human teratomas.

Cysts lined with stratified squamous epithelium and containing exfoliated keratinized cells are common. It was mentioned that epithelium of this type is frequently continuous with columnar or cuboidal epithelium with goblet cells. This is interpreted as an indication that the 2 types of epitheliums are derived from a common prototype. Willis (*2*) stated that much, and perhaps all, of the squamous epithelium in his teratomas arises from glandular elements through metaplasia. We do not feel it is necessary to assume that this type of metaplasia occurs in our material.

It seems more probable that undifferentiated cells may give rise directly to both goblet-celled and stratified squamous epitheliums. We believe that this is indicated by the close proximity of the 2 types in cysts.

Cartilage, bone, fat, and muscle are found in a large percentage of our mouse tumors and in teratomas in man. Hepatic, renal, and lung tissues and teeth are inconspicuous or absent in both human and mouse testicular teratomas. Endocrine tissues, with the possible exception of adrenal medulla, are seen infrequently.

About one fourth of strain 129 teratomas contains undifferentiated "embryonal" cells, which are believed to give rise to both undifferentiated and differentiated cells. In studies of a transplantable ovarian teratoma of the mouse, Jackson and Brues (*13*) concluded that " . . . the most immature cells are responsible for the growth of this tumor, since they contain all of the mitotic activity." Fekete and Ferrigno (*14*) arrived at a similar conclusion based upon their study of a transplantable ovarian teratoma of the mouse. According to these investigators, the pluripotential nature of the embryonic cells seemed probable.

Because some strain 129 teratomas do not continue progressive growth indefinitely and in tumors of older animals embryonal tissue is usually absent, it is assumed that in most cases the embryonal cells differentiate and cease proliferation. In this respect, some of these mouse tumors are like those described in the horse by Willis and Rudduck (*8*) rather than than like those described in man.

Most of the testicular teratomas of old mice of strain 129 can be classified as adult-type growths composed predominantly of differentiated tissues. In their growth and differentiation tendencies, most of the mouse tumors described here seem to resemble the adult teratomas found in the horse (*7,8*) and the ovarian and extragonadal teratomas in man (*2,10*). Teratomas of the human testis, however, are generally malignant and contain much actively growing immature tissue (*11,4,10, et al.*). Most strain 129 tumors also differ from their murine ovarian homologues, which are described as large, progressively growing, and in most cases, readily transplantable tumors (*13,14*). It might appear that we are dealing with 2 fundamentally different types of growths, one adult, the other embryonal. However, Willis (*2*) expressed the opinion that no sharp distinction can be made between adult and embryonal teratomas. He suggested that ". . . the predominance of growths of a particular kind in a particular kind of situation is probably no more than the consequence of a particular kind of nidus." Our observations support this contention. The teratomas that develop in the genetically homogeneous strain 129 mice vary somewhat in their structure and more radically in their capacity for progressive growth. Environmental rather than genetic influences in these animals must determine whether a teratoma will develop and, furthermore, whether it will be of adult or embryonal type, capable of further proliferation. Friedman and Moore (*4*) have cautioned against designating "adult" teratomas with no histological recognizable malignant components as "benign" growths. Metastases of human testicular

teratomas that appeared to contain only adult tissues have been reported (*11*).

Even though no organs are formed in strain 129 teratomas, certain tissue interrelationships are observed. As in human tumors (*2*), almost all smooth muscle is associated with goblet-cell epithelium. The development of this relationship can be traced through various stages, from an epithelial vesicle with mesenchyme oriented around it to characteristic alimentary or respiratory mucosa encircled by connective-tissue and smooth-muscle layers.

An inductive relationship between nervous tissue and cartilage, similar to that reported by Willis, probably exists but cannot be demonstrated in the mouse tumors because of the ubiquity of nervous tissue.

As to the state of organization of tissues within strain 129 teratomas, the results of the present study agree with those of numerous other workers (*2,15,13 et al.*). The mouse teratomas are not distorted fetuses; they do not exhibit signs of axiation, segmentation, or true organs; they contain an anachronous mixture of tissues of various degrees of maturity, and some grow progressively.

Willis (*2*) pointed out that in human testicular teratomas, mucous glands and goblet-celled epitheliums secrete mucus. He believed that the richly cellular red bone marrow was capable of blood-cell formation. Some of the large cysts found in strain 129 teratomas contain a mucinous secretion, and the red bone marrow in these tumors appears to be functioning hematopoietically. The teratomas in the testes of mice immediately after autopsy frequently exhibit twitching and pulsating movements that correspond with the histologic findings of abundant striated skeletal and cardiac muscle. Pulsations, twitches, and peristaltic contractions have been observed in fragments of a transplantable form of these teratomas cultivated *in vitro*. Hair follicles produce hair, cells of adipose tissue accumulate large vacuoles of fat, and it is likely that some of the well-formed nerve fibers conduct impulses.

Several reports in the literature present evidence that tumors occur much more frequently in ectopic than in scrotal testes in man. According to Ewing (*3*) teratoma testis is ". . . particularly prone to develop in atrophic, undescended organs." Gilbert and Hamilton (*16*) emphasize that ectopy is not necessarily the cause of testicular cancer, but congenital defects such as inguinal hernia, pseudohermaphroditism, and ectopy often accompany neoplasia of the testis. Dixon and Moore (*6*) report that, of 24 tumors in undescended testes found in army personnel, 17 were seminomas, which indicates a marked predisposition toward this type of tumor in ectopic testes. Aside from the fact that strain 129 teratomas exist at birth, when the mouse testis is normally in abdominal position, abnormalities that might later result in cryptorchidism or herniation are extremely rare. Furthermore, abnormalities of the reproductive system, other than teratoma testis and small testis, are rare in these mice. There does not seem to be a causal relationship between undescended testes and teratoma testis in strain 129 mice. On many occasions teratomas have

been observed in the scrota of living mice, but of those undetected until autopsy, none of the testes were unable to descend for reasons other than their large size.

It has been claimed that teratomas of the human testis develop in the rete (*3*). In several cases in strain 129 teratomes, the rete was separated from rather than part of the tumor. From our observations on serial sections of teratomatous testes few, if any, teratomas of strain 129 mice develop in the rete.

It has been suggested that small testis and teratoma testis may have etiologic factors in common—both occur in strain 129 mice, both are congenital, both show a preference in laterality, and both involve hemorrhage (*1*).

There are many similarities in the structure and behavior of testicular teratomas as they exist in man and the mouse. The types, arrangements, and degrees of differentiation of tissues are not conspicuously different in the teratomas of the 2 species. Willis (*11*) suggested that human testicular teratomas may be congenital; and testicular teratomas are found in newborn mice. In our opinion the failure of mouse teratomas to metastasize does not indicate that they are fundamentally different from their human counterpart. In view of the similarities, and in the absence of evidence to the contrary, it seems likely that testicular teratomas in man and the mouse are basically alike. The discovery of information leading to a better understanding of the nature of teratomas in mice may contribute to knowledge about their homologues in man as well.

Summary

The testicular teratomas of strain 129 mice described are congenital. The types of tissues and their chaotic arrangement are similar to those found in homologous human and equine tumors of the testes and in murine ovarian teratomas. The majority of testicular teratomas found in mice 20 to 30 days of age are composed entirely of differentiated, adult-type tissues, but approximately one third have embryonic and immature components. The proportion of tumors containing embryonic and immature cells decreases with advancing age of the host.

When grafted subcutaneously, 4 of 19 strain 129 testicular teratomas grew progressively for many transplant generations. Considering the embryonic nature and transplantability of some of these tumors, they are similar in their behavior to the malignant teratomas that develop in the testes of human beings and in the ovaries of mice. In other grafts, tissues differentiated completely and did not grow progressively as transplantable tumors. These resemble the usually benign teratomas of the horse testis and those of the ovary and extragonadal sites in man.

Evidence is presented that the rete testis is not involved in neoplastic change. It is believed that there may be common etiologic factors operating in the production of these tumors and another congenital testicular abnormality, small testis, which results from degeneration of the sperm-

atogenic tubules. The tumors are not associated with other abnormalities of the reproductive system.

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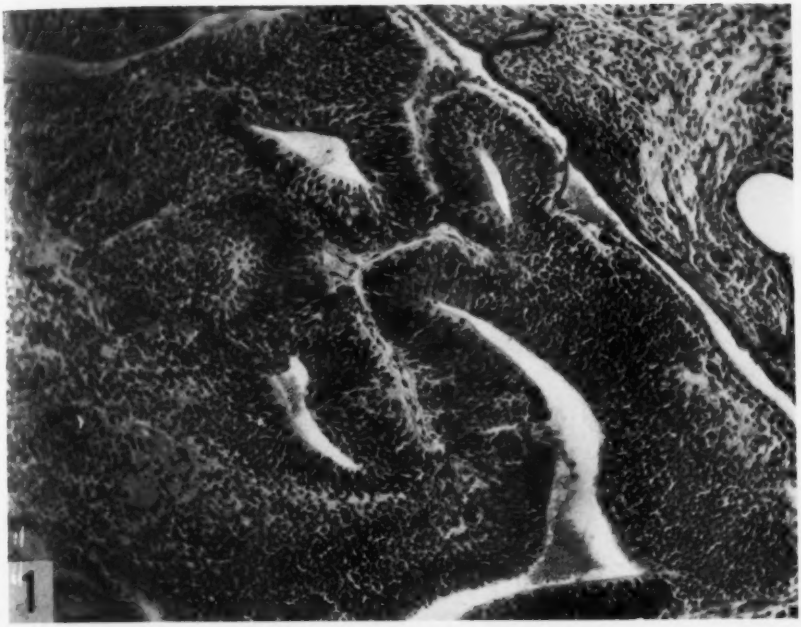
Plates 56 through 63 show cells and tissues in teratomas of mice 20 to 30 days of age. Plate 64 shows sections from teratomas of older mice.

Plate 56

Figure 1.—Immature neuroepithelium lining distorted tubules. *Note* ependymal layer with mitotic figures. In some places the neural tissue is bounded by an external limiting membrane. In other places, the cells of the mantle layer spread out and merge with more differentiated neuroglia.

Figure 2.—Nervous tissue containing large cell bodies like those of motor neurons.



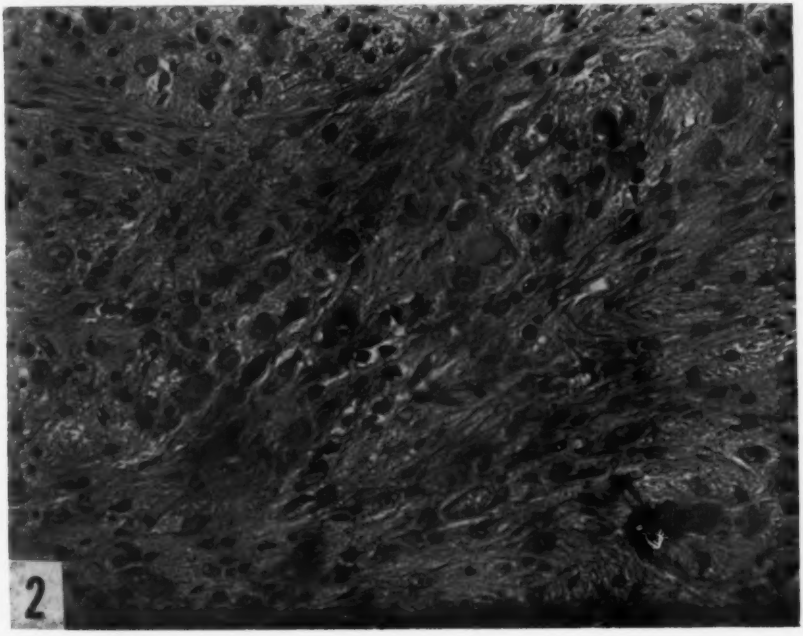


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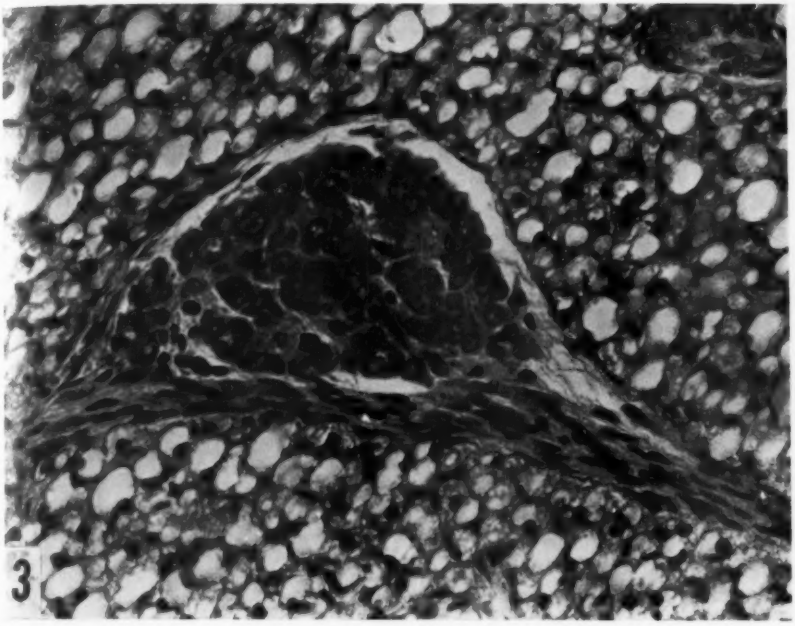
PLATE 57

FIGURE 3.—A ganglion with nerve fibers embedded in fat.

FIGURE 4.—Epitheliums of several types lining cyst. *Note* mucus-secreting columnar, stratified squamous, pseudostratified columnar, and ciliated cuboidal epitheliums contiguous with one another.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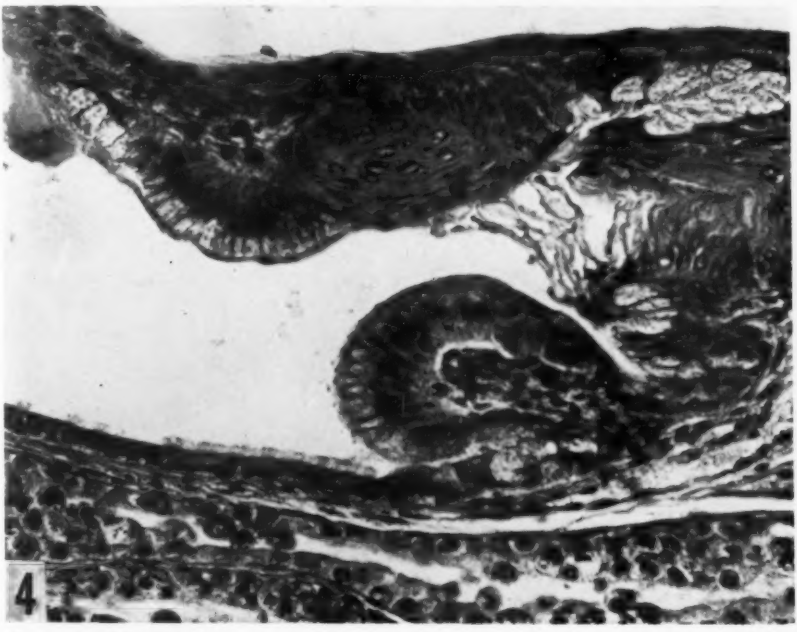
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PLATE 58

FIGURE 5.—Gland resembling islet-free pancreas or parotid gland. *Note* also bone with red marrow, adipose tissue, and skeletal muscle in cross and longitudinal section.

FIGURE 6.—Mixed gland with serous and mucous cells connected by a duct to a cyst lined by respiratory epithelium. *Note* also skeletal muscle and cuboidal epithelial cysts.

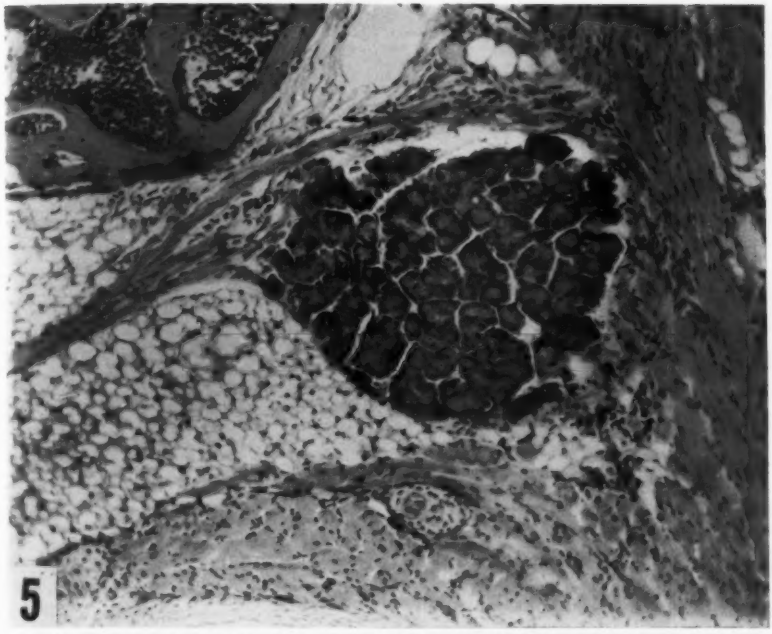
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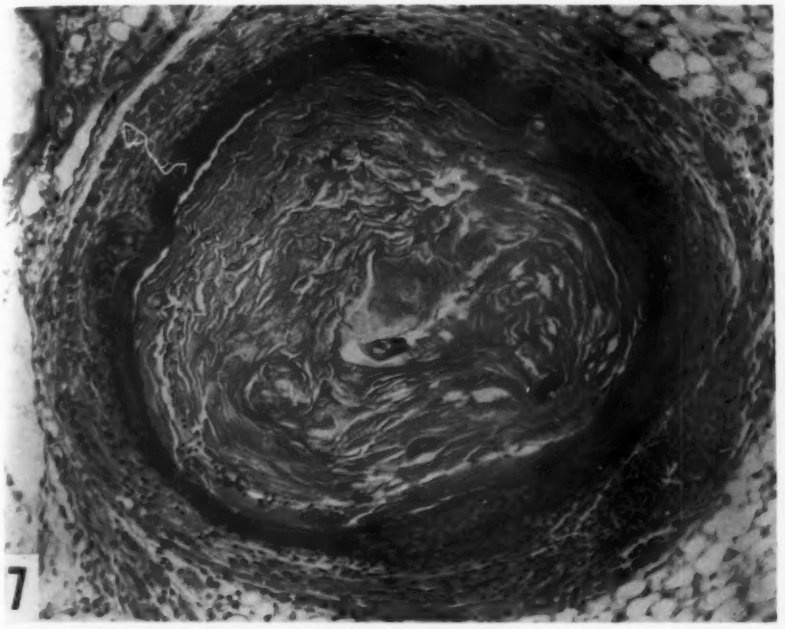
PLATE 59

FIGURE 7.—Cyst lined by stratified squamous epithelium of epidermal type. *Note* sebaceous glands, encircling smooth muscle, and desquamated keratinized epithelial cells.

FIGURE 8.—Epithelial cysts adjacent to hair follicles, fat, striated muscle, and cartilage.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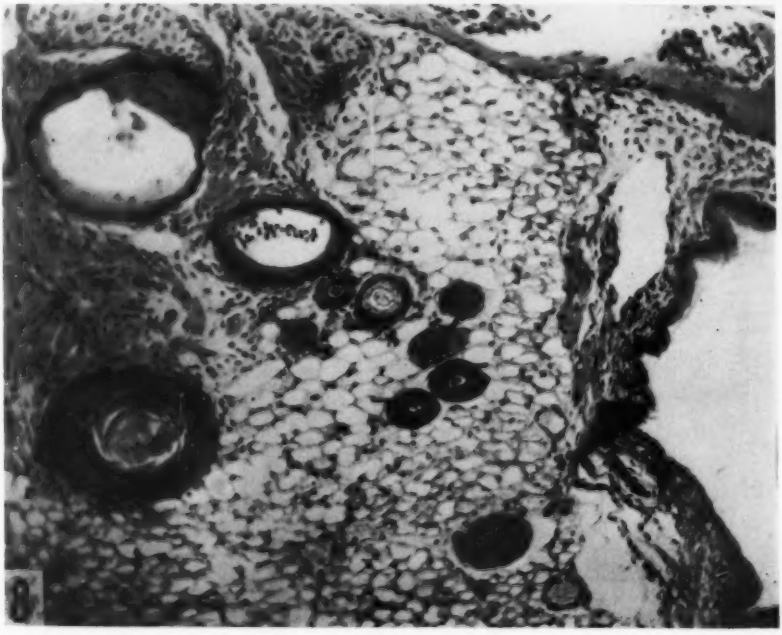
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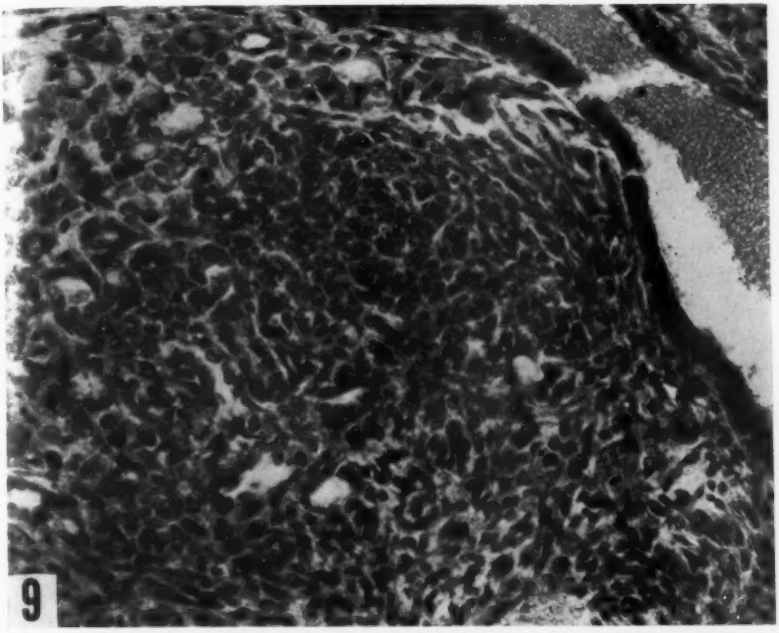
PLATE 60

FIGURE 9.—Embryonic mesenchyme showing mitotic activity and an adjacent epithelial cyst.

FIGURE 10.—Endochondral ossification and red bone marrow. *Note* degenerating cartilage cells, osteoblasts, megakaryocytes, and immature blood cells. At the *lower left* are spermatogenic tubules.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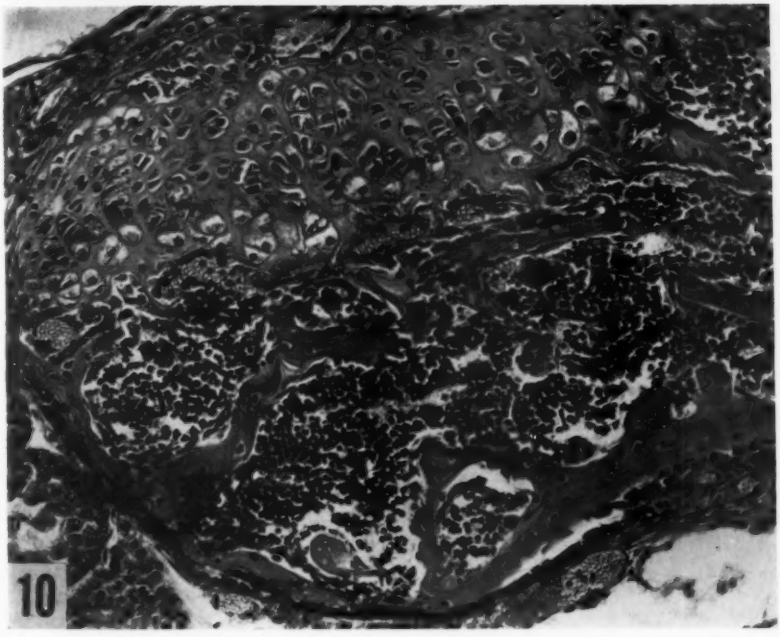
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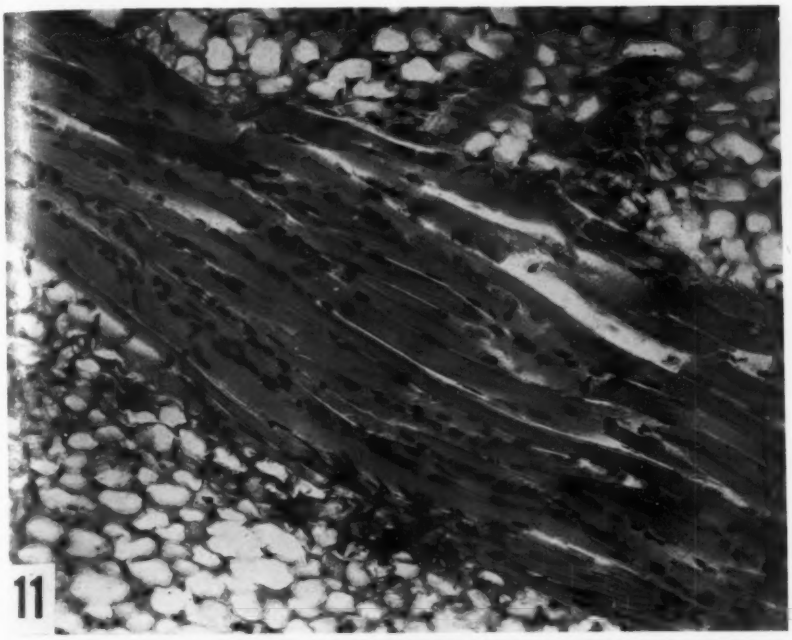
PLATE 61

FIGURE 11.—Bundle of skeletal muscle fibers embedded in adipose tissue.

FIGURE 12.—Smooth-muscle layers encircling a cavity lined by respiratory epithelium. Glands drain into the cavity. Immature cardiac muscle, simple cuboidal epithelial cysts, an island of cartilage, and spermatogenic tubules are also shown.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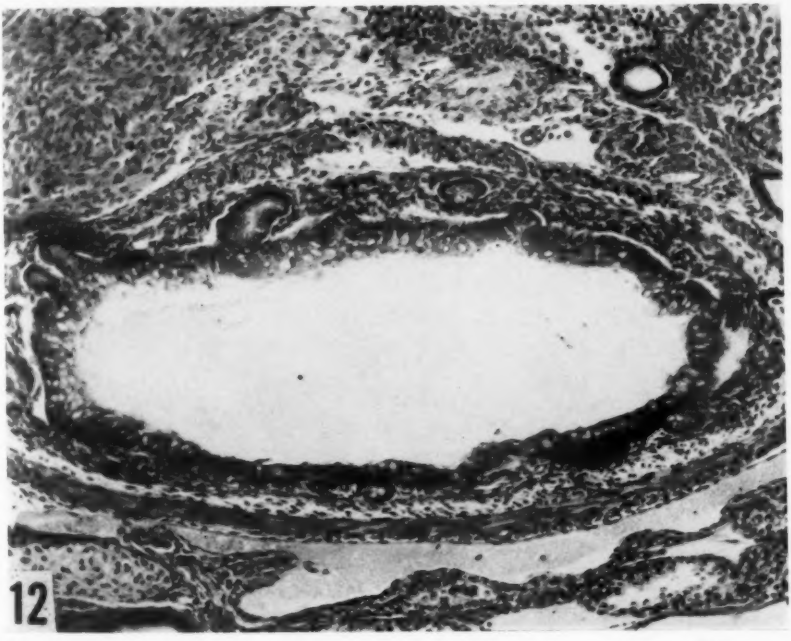
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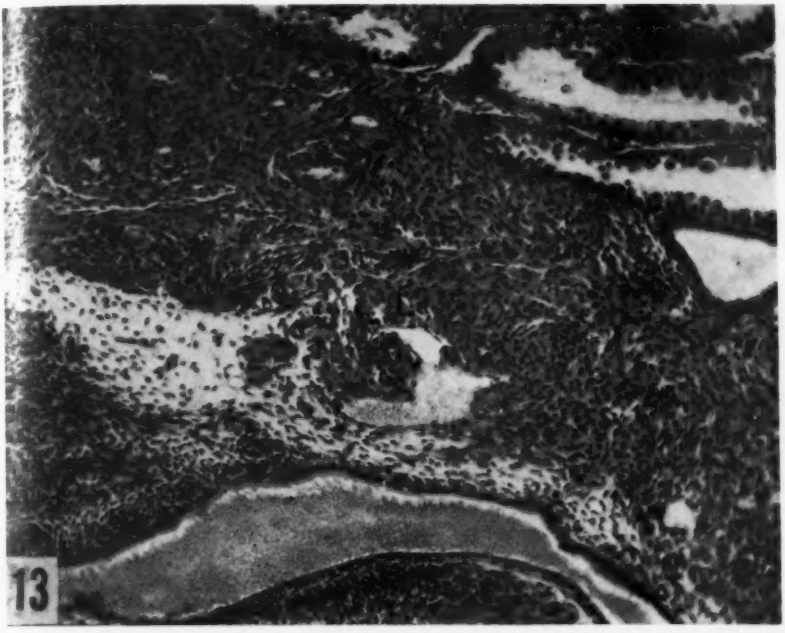
PLATE 62

FIGURE 13.—Fetal cardiac muscle, simple cuboidal epithelium, and spermatogenic tubules.

FIGURE 14.—Nodule resembling an endocrine gland adjacent to fat, tubuloalveolar glands, and a cyst lined with cuboidal ciliated cells. In addition, skeletal muscle attached to bone and spermatogenic tubules are shown.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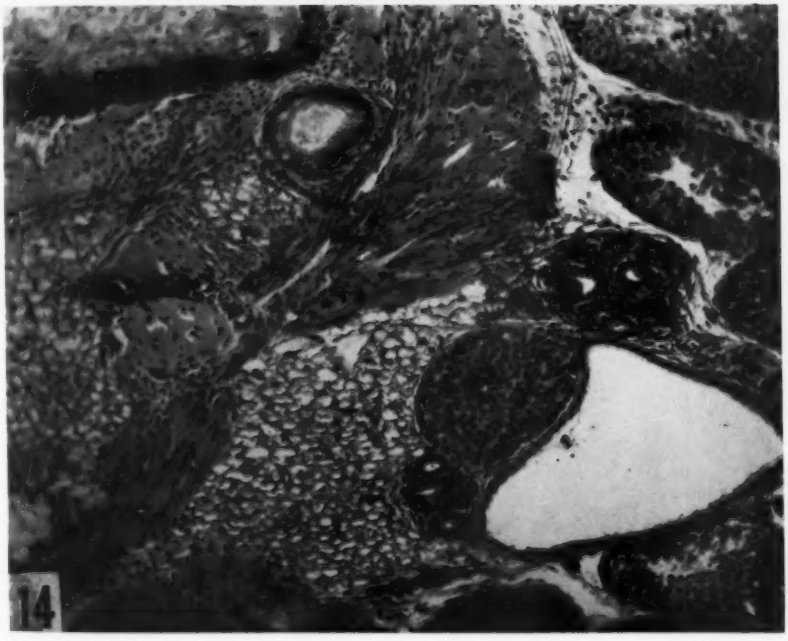
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PLATE 63

FIGURE 15.—Clusters resembling notochord and adrenal medulla. These clusters are surrounded by membranes, and some cells are dividing.

FIGURE 16.—A cluster of cells, resembling notochord and adrenal medulla, in which there is a spicule of bone. Epithelial cysts, nervous tissue, cartilage, bone, and red bone marrow are also shown.

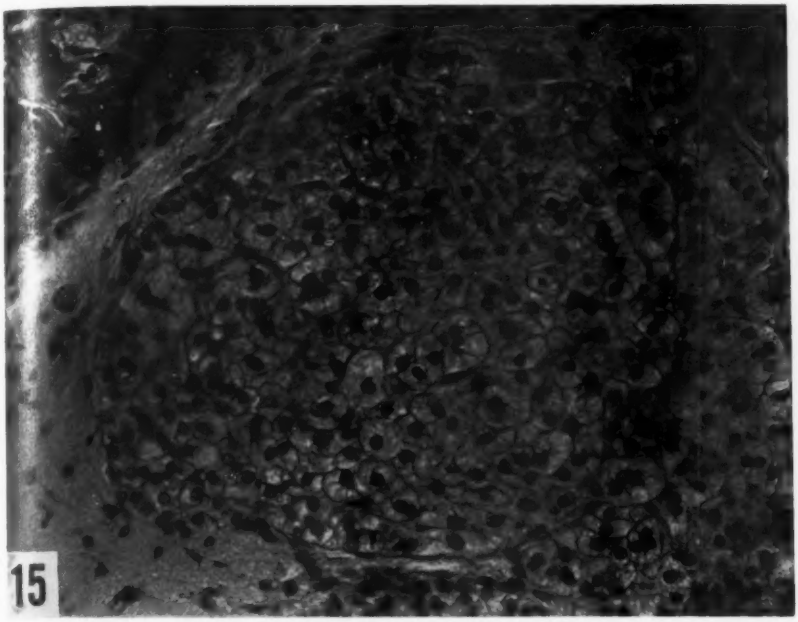
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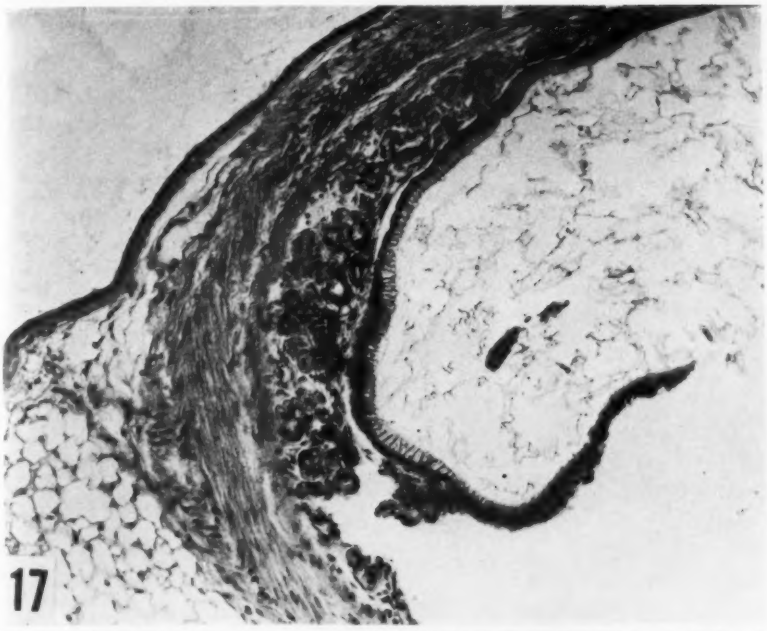
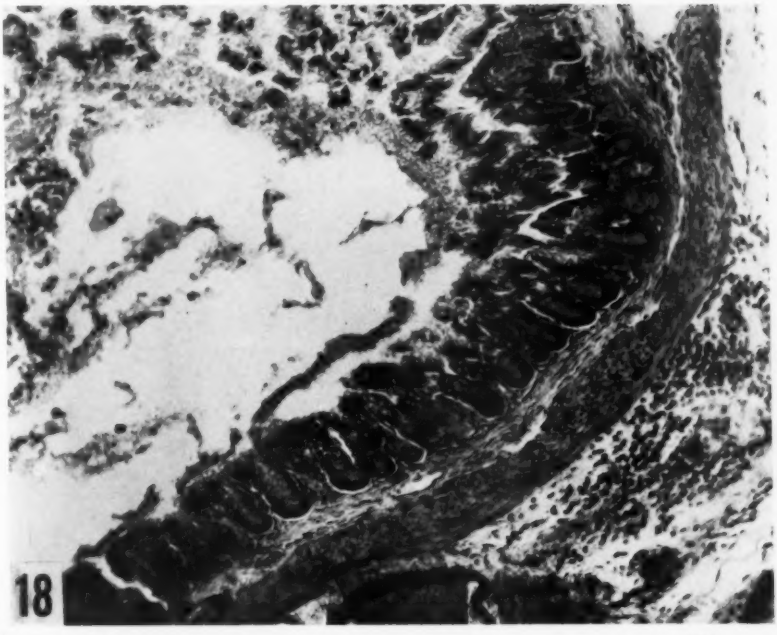
PLATE 64

FIGURE 17.—Cyst lined by respiratory epithelium with glands and surrounding layers of smooth muscle.

FIGURE 18.—Structures resembling intestinal villi in a teratoma of an old mouse. *Note* surrounding layers of smooth muscle.





420678—57——15

Deoxypentose Nucleic Acids and Nucleoproteins of Malignant Tissues. III. Electrophoretic Studies of the Deoxypentose Nucleic Acids of Lymphoma #1 and of Calf Thymus [1]

JOSEPH SHACK *and* ROBERT J. JENKINS,[2] *National Cancer Institute,*[3] *Bethesda, Maryland*

Previous papers in this series (*1,2*) have dealt with the isolation and some physicochemical properties of deoxypentose nucleic acid (DNA) from transplantable lymphoma #1 of the strain A mouse. As part of the characterization of this material, an investigation of its electrophoretic properties was carried out over a wide range of *p*H values. It was evident that the dependence of electrophoretic mobility on *p*H found with the lymphoma DNA was significantly different from that reported for calf-thymus DNA by Creeth, Jordan, and Gulland (*3*). They had, however, used a different series of buffers than the monovalent buffer systems used in our work. To determine whether these results were indicative of real differences between the electrophoretic properties of the 2 DNA's, parallel studies were carried out on calf-thymus DNA under exactly the same conditions used with lymphoma DNA. Because of the larger amount of thymus nucleate available, our data on this DNA are somewhat more extensive than on the lymphoma DNA. In addition, the effects of bivalent anions on mobility were also investigated with thymus nucleate. Our data show that, under comparable conditions, the nucleates of calf thymus and mouse lymphoma do not differ significantly in their electrophoretic properties.

Materials and Methods

The preparation of the samples of DNA have been described elsewhere (*2,4*).

The electrophoretic experiments were carried out at 1° C. in the usual manner with an Aminco-Stern Model L electrophoretic assembly. For the most part standard 11 ml. single-section electrophoretic cells were used; a few experiments were carried out in the American Instrument Company clinical cell for reasons that will be discussed later. In general, 4 or 5 positions of the boundaries were determined over a period of 2 to 3 hours.

[1] Received for publication February 6, 1957.

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The monovalent buffers used were citrate (below *p*H 3.6), acetate, cacodylate, veronal, borate, and glycine; in all cases the cation was sodium. At very high *p*H values, mixtures of NaOH and NaCl were used.

In most cases the *p*H of the solutions was measured at room temperature and corrected to 1° C. by use of the temperature coefficients of the dissociation constants given by Harned and Owen (*5*). When such data were not available *p*H measurements were made at 1°, the electrode system being calibrated with standard buffers whose *p*H values at that temperature are known [*cf.* Bates (*6*)]. At high *p*H values, solutions of carbonate-free NaOH were used as standards; their *p*H values were calculated from the activity coefficients and the values of Kw given by Harned and Owen (*5*).

In most cases the solutions for electrophoretic analysis were made up by weight in water,[4] cooled to 1° C., and equilibrated by dialysis against the desired buffer. In some experiments, however, the solution of nucleate was cooled, diluted with an equal volume of cooled double-strength buffer, and run against a similarly prepared buffer without dialysis , in the manner described by Creeth, Jordan, and Gulland (*3*). Results from the 2 methods of preparing the solutions were in agreement.

The concentration of DNA was in every case 0.16 percent or less. Even at this low concentration some sticking of the viscous DNA solution on the surface of the descending limb of the cell occurs during compensation. This can easily be seen if a colored material is added to the solution of DNA. As a result of this sticking, the descending boundary appears at first to have been compensated further than the ascending one, but as the DNA solution flows down along the surface after compensation, the descending boundary gradually rises to the correct level and then remains stationary. This phenomenon results in an initial retardation of the downward movement of the descending boundary when electrophoresis is started before the boundary has risen to its correct level; no such effect occurs, however, on the ascending side. To minimize errors due to this sticking effect in the determination of mobilities from the descending patterns, slow rates of compensation were used, and the descending boundary was in every case allowed to reach its stationary level before application of the voltage. When these precautions were observed, the rates of movement of both boundaries were constant and in close agreement. As a further check, a number of experiments were done in the Aminco clinical cell in which no compensation is required. Although the initial boundaries are not so sharp, the mobility values were the same as those obtained in the 11 ml. single-section cells.

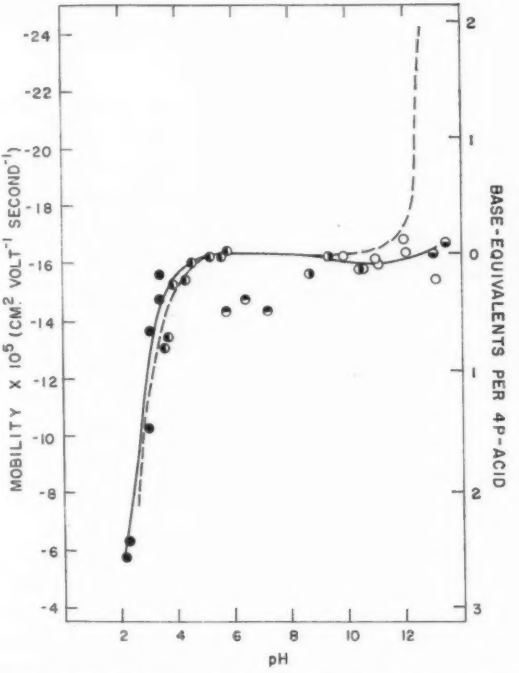
Results and Discussion

The results on calf-thymus DNA showed, in agreement with those of others (*3,8–13*), only a single boundary at all the *p*H values and in all the buffers used. The mouse-lymphoma DNA also exhibited only a single boundary at all conditions.

[4] At the concentration of nucleate used in the present study the "water denaturation" discovered by Thomas (*7*) does not occur.

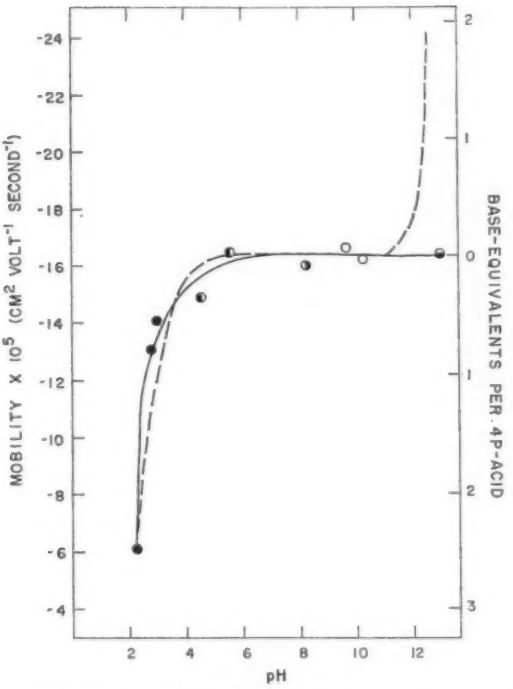
The mobility-*p*H curves of the 2 nucleates are given in text-figures 1 and 2. The values of mobility were taken from ascending patterns because of their greater sharpness and because the motion of the ascending boundary appears to be unaffected by the "sticking effect." Mobility-*p*H curves constructed from descending mobilities were, however, not significantly different from those shown here. The mobility-*p*H curves of the 2 nucleates are practically the same; it is evident, therefore, that these 2 nucleates cannot be differentiated on the basis of the relationship of electrophoretic mobility to *p*H. This result suggests that electrophoresis will not prove to be a very sensitive method for the differentiation of deoxyribonucleic acids.

The mobility-*p*H curves are compared in text-figures 1 and 2 with forward-titration curves that were determined at 3° C. The scale of the titration curves is adjusted so that each equivalent of acid or base bound per 4P corresponds to a mobility change of 4.07. Below *p*H 11, a close correspondence is found between titration and mobility-*p*H curves. No



TEXT-FIGURE 1.—Variation of electrophoretic mobility of deoxypentose nucleate of calf thymus with *p*H at 0.10 ionic strength. Mobilities were calculated from the ascending patterns. DNA = 0.16 percent. Buffers: ●, citrate; ◐, acetate; ◓, cacodylate; ◑, borate; ○, glycinate; ◒, NaOH-NaCl.

The *dotted curve* is the electrometric forward-titration curve that is obtained on addition of acid or alkali to a solution of calf-thymus DNA in 0.1 *M* NaCl at 3° C. Ordinate, corrected equivalents of acid, or base bound per 4P.



TEXT-FIGURE 2.—Variation of electrophoretic mobility of deoxypentose nucleate of mouse lymphoma with *p*H at 0.10 ionic strength. Mobilities were calculated from the ascending patterns. DNA = 0.16 percent. Buffers: ●, citrate; ◐, acetate; ◑, borate; ○, glycinate; ◒, NaOH-NaCl.

The *dotted curve* is the electrometric forward-titration curve of lymphoma DNA in 0.1 *M* NaCl at 3° C. Ordinate, corrected equivalents of acid, or base bound per 4P.

change is observed between *p*H 5 and 11, a region in which no titration occurs. The sharp drop of mobility below *p*H 4 is in agreement with other reports (*8–10,3*) and occurs in that region of *p*H where the negative charge should decrease as the amino groups are titrated. Our results are in agreement with the conclusion of Stenhagen and Teorell (*8*) that below *p*H 10 the mobility-*p*H curve is similar to the titration curve.

The absence of a rise of mobility above *p*H 12 is surprising, since it is to be expected that titration of the enolic groups in this region would result in a substantial increase of the negative charge borne by the nucleate. However, since initial titration of the enolic groups results in an irreversible denaturation of DNA (*14,15,2*), it can only be concluded from such a result that the mobility of denatured DNA at high *p*H is nearly the same as that of undenatured DNA in its zone of stability. It is possible that changes of shape, ion binding, and degree of hydration occur with denaturation, and that the effects of these changes nearly

compensate the effect of increased negative charge; an exact evaluation of the influence of such factors is not possible at present.

An effect of titration of enolic groups on mobility can, however, be demonstrated with DNA that had previously been denatured by exposure to high *p*H (*p*H 12.2 at room temperature). With such alkali-treated DNA the titration of enolic groups occurs reversibly in the range *p*H 9 to 12, and results are uncomplicated by irreversible processes. The mobilities with the alkali-denatured DNA (of thymus) were (at 0.1 ionic strength) −16.3 at *p*H 12.9 (NaOH-NaCl), −14.2 at *p*H 10.2 (glycine), −13.5 at *p*H's 9.2 and 8.3 (borate), and −13.9 at *p*H 4.6 (acetate). Thus, the alkali-treated DNA exhibits change of mobility in that region of *p*H where titration of its enolic groups occurs (*14,2*). It is to be noted that the mobility of alkali-treated DNA is lower than that of undegraded DNA under the same conditions, a finding in agreement with the report of Ui and Watanabe (*16*).

It should also be noted that the initial titration of the amino groups, as well as of the enolic groups of DNA, leads to irreversible denaturation (*15*). The values of mobility given in text-figures 1 and 2 for *p*H values below *p*H 4 undoubtedly refer to DNA that has to some extent been denatured by exposure to low *p*H. It was observed, however, that the mobility of DNA that had been previously denatured by exposure to acid (*p*H 2.2) was, in the neutral region, close to that found with alkali-denatured DNA. It appears, therefore, that most of the drop of mobility below *p*H 4 results from titration rather than from denaturation.

The reduction of mobility that results from denaturation by alkali or acid (from *ca.* 16.4 to 13.5–14) is very much smaller than the reduction of the specific viscosity of the solution (to a few percent of its original value). This fact is in harmony with the conclusion of Stenhagen and Teorell (*8*) and of Creeth *et al.* (*3*) that the effective viscous-retarding force is determined by the viscosity of the solvent rather than of the solution. This conclusion was based on findings (*8,3*) that the electrophoretic mobility of DNA changed only slightly over a several-fold range of DNA concentrations even though the specific viscosity of the solutions varied several fold. We have obtained similar results with our preparation of DNA.

Inspection of text-figure 1 reveals certain differences among the series of univalent anions that were used, although in no case was any variation with *p*H observed with a given anion (above *p*H 5). Thus, mobilities in acetate, chloride, borate, and glycinate were nearly the same. Mobilities in cacodylate were lower, while those in citrate were higher than those in acetate at the same *p*H. Likewise, Creeth *et al.* (*3*) have stated that ". . . citrate gave high values, borate low." Some experiments in phosphate and carbonate buffers were also carried out with thymus DNA to test the effect of bivalent anions on mobility. Results are given in table 1. These mobility values may be compared with those obtained when the ionic strength was varied by changing the concentration of NaCl in the presence of 0.01 *M* sodium veronal buffer, *p*H 7.6; mobilities

($\times 10^5$) were −19.6, −16.6, −15.5, and −14.4 at ionic strengths of 0.05, 0.10, 0.20, and 0.30, respectively. It is evident that the mobilities in phosphate buffers are higher than in monovalent buffer systems, whether compared at the same ionic strength or at the same concentration of Na^+. The series in carbonate were all at 0.10 ionic strength, and in this case a substantial rise with increasing *p*H is seen. However, no increase of mobility is found between *p*H 11.4 and 12.95, even though both the titration of enolic groups and the irreversible loss of viscosity occur in this region. It therefore appears that the rise of mobility between *p*H 9.1 and 11.4 observed in the carbonate buffers is due to specific ion effects rather than to any increase of negative charge resulting from titration of the enolic groups of the DNA. The effects of bivalent ions may possibly result from some degree of binding between such anions and the DNA (*17,18*).

TABLE 1.—*Effect of bivalent anions on mobility*

Buffer	*p*H	Conc. $H_2PO_4^-$ or HCO_3^- (M *per l.*)	Conc. $PO_4^=$ or $CO_3^=$ (M *per l.*)	Conc. NaOH (M *per l.*)	Total Na^+ (M *per l.*)	Ionic strength	Mobility $\times 10^5$
Phosphate	5.7	0.093	0.007	—	0.107	0.114	−17.9
Phosphate	7.6	0.024	0.176	—	0.183	0.275	−17.2
Carbonate	9.1	0.085	0.005	—	0.095	0.10	−14.8
Carbonate	10.3	0.025	0.025	—	0.075	0.10	−15.5
Carbonate	10.9	0.0077	0.0924	—	0.0693	0.10	−18.0
Carbonate	11.4	0.0025	0.0325	—	0.0675	0.10	−18.5
Carbonate	12.95	—	0.0267	0.02	0.0734	0.10	−18.2

Summary

An investigation of the electrophoretic properties of deoxypentose nucleates of calf thymus and of strain A mouse lymphoma #1 is reported.

Both nucleates exhibited a single electrophoretic boundary at all *p*H values from about 2 to 13 and in all the buffer systems used. The variation of electrophoretic mobility with *p*H was determined over this range in a series of buffers containing only monovalent ions; the mobility-*p*H curves of the 2 nucleates were practically identical under these conditions.

Below *p*H 11.6, the mobility-*p*H curves closely paralleled the forward-titration curves. Above *p*H 11.6, no rise of mobility was observed in the region of *p*H where titration of enolic groups occurs; possible explanations for this lack of correspondence were discussed.

Electrophoretic studies in buffers containing bivalent anions indicate that discrepancies between electrophoretic and titration behavior may arise from specific effects of the bivalent anions, possible as a result of their binding by the nucleate.

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Control of Mouse Pox (Infectious Ectromelia) by Immunization with Vaccinia Virus[1, 2, 3]

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Mouse pox (infectious ectromelia) is a disease of mice first described by Marchal (*2*), who demonstrated that it was caused by a virus and that infected tissues often contain eosinophilic, intracytoplasmic inclusion bodies. She named it infectious ectromelia because of loss of the extremities in some infected mice. The disease occurs in 2 forms, an acute visceral form associated with a high mortality rate and a chronic form associated with cutaneous lesions of the tail, feet, and body. Burnet (*3,4*) first demonstrated the close immunologic relationship between the virus of infectious ectromelia and vaccinia virus. He recognized ectromelia virus as the mouse-specific member of the family of mammalian pox viruses and suggested the more appropriate designation "mouse-pox" for the disease. The disease has been carefully studied by Fenner in Australia (*5*).

Mouse pox is enzootic in laboratory mice in Europe, but until recent years it has not been a serious problem in the United States. An epizootic disease fitting the description of mouse pox made its appearance in laboratory mice at Yale University School of Medicine between 1946 and 1950. In the summer of 1951 this disease appeared for the first time in the several mouse colonies of the Department of Anatomy. It was definitively identified as mouse pox in the authors' colony by: *a*) observation of the clinical course of the disease, *b*) isolation and characterization of the causative virus, *c*) successful cross-immunization against the isolated virus by prior inoculation with vaccinia virus, *d*) inhibition of vaccinia hemagglutinin by the sera of some mice that survived the disease, *e*) demonstration of eosinophilic intracytoplasmic inclusion bodies in tissues of some infected mice and in chick chorio-allantoic membranes (*1,6,7*).

Because of the continuing presence of other infected mouse colonies in the immediate environment it was decided not to exterminate the surviv-

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[2] Preliminary report presented at the meetings of the Federation of Societies for Experimental Biology and Medicine, held in March, 1955 (*1*).

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ing mice but to attempt to control the disease by cross-immunization with vaccinia virus.

Materials and Methods

Isolation and identification of the ectromelia virus used has been described previously (*6,7*). The virus was propagated on the chorioallantoic membrane of 12-day-old embryonated eggs incubated for 3 days at 35 to 36° C. before harvesting. Membranes were ground with 90-mesh alundum, and 1 ml. of saline was added for each membrane. The material was centrifuged, and to each ml. of supernatant were added 2 mg. of crystalline penicillin G potassium, 20 mg. of streptomycin sulfate, and, finally, an equal volume of 50 percent glycerin. Activity was maintained for many months during storage at 4° C. Mice were challenged by subcutaneous inoculation of 0.02 cc. of this preparation into one of the hind feet.

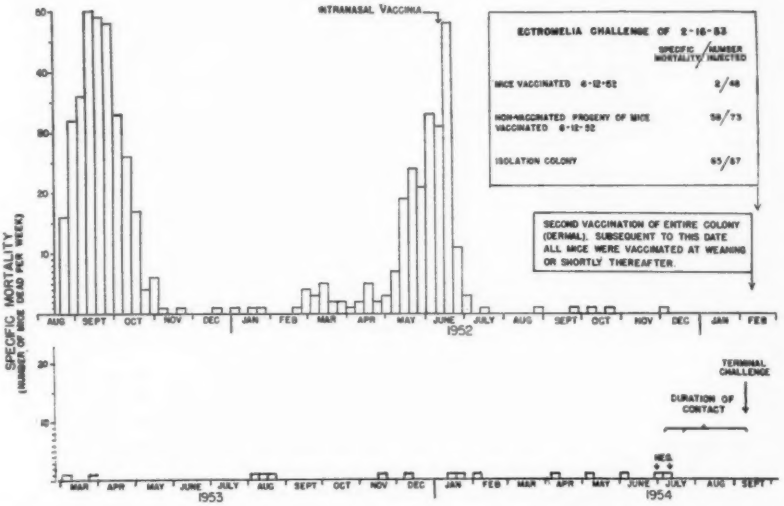
Vaccinia virus of the Nelson and Levaditi strains and commercial calf-lymph vaccine were used. All were propagated at 37° C. on 12-day-old embryonated eggs. Preparation and storage were similar to that for ectromelia virus.

During the period covered by this report, the authors' colony contained mice of the following strains: A, C3H, CBA, BC, C57, (C3H × A)F_1 hybrids, (CBA × A)F_1 hybrids, Af, C3Hf, and CBAf. The latter 3 represent the A, C3H, and CBA strains foster-nursed on the C57 strain. Breeding stock was obtained initially from Dr. W. U. Gardner and Dr. L. C. Strong. The IP Br, 3 Ca Mg, and "Briody" mice of table 1 were not part of the breeding colony. The former 2 represent single pens of mice obtained from Dr. Strong. The "Briody" mice were commercially available albino mice obtained from Dr. B. A. Briody.

Observations

Mortality from mouse pox in the authors' colony is shown in text-figure 1. Mice that died during the first 4 months, a period of high mortality, showed only the acute visceral form of the disease (fig. 1). After the death rate had returned to a low level, cutaneous lesions appeared among the surviving mice. Starting in February, 1952, the mortality rate began to climb again as a result of recurrence of the acute visceral form of the disease. Only the progeny of the survivors of the first outbreak were involved (no new mice had been introduced into the colony). When the mortality rate had again risen to serious proportions, it was decided to attempt to control the disease by cross-immunization with vaccinia virus. On June 1, 1952, the authors' colony was removed from the common mouse quarters of the Department of Anatomy to a separate animal room in the new Biological Laboratory, which contained excellent facilities for isolation and cage sterilization. On June 12, 1952, all mice older than weaning age were vaccinated by intranasal instillation, under ether anesthesia, of a 1 : 100 dilution of the Levaditi strain of vaccinia virus. Pilot experiments had indicated that intranasal instillation in mice of

large doses of vaccinia virus might in itself cause mortality, some strains of mice being more susceptible than others (table 1). The 1 : 100 dilution of vaccinia virus was chosen as the largest dose of virus that could be administered intranasally to the sensitive BC strain without in itself causing mortality. During the next 10 days, mortality from mouse pox continued unabated. Consideration of the incubation period of mouse pox would indicate that most if not all of these deaths were in mice already infected with ectromelia virus at the time of vaccination. After the 10th day, mortality from mouse pox declined sharply (text-fig. 1). Of the 15 mice that died of mouse pox between the 10th and 38th days after


INTRANASAL VACCINIA
ECTROMELIA CHALLENGE OF 2-16-53
SPECIFIC MORTALITY / NUMBER INJECTED
MICE VACCINATED 6-12-52 2/48
NON-VACCINATED PROGENY OF MICE VACCINATED 6-12-52 58/73
ISOLATION COLONY 65/67
SECOND VACCINATION OF ENTIRE COLONY (DERMAL). SUBSEQUENT TO THIS DATE ALL MICE WERE VACCINATED AT WEANING OR SHORTLY THEREAFTER.
SPECIFIC MORTALITY (NUMBER OF MICE DEAD PER WEEK)
AUG SEPT OCT NOV DEC JAN FEB MAR APR MAY JUNE JULY AUG SEPT OCT NOV DEC JAN FEB
1952
TERMINAL CHALLENGE
DURATION OF CONTACT
NEG.
MAR APR MAY JUNE JULY AUG SEPT OCT NOV DEC JAN FEB MAR APR MAY JUNE JULY AUG SEPT
1953
1954

TEXT-FIGURE 1.—Mortality from mouse pox in the authors' colony, as determined by gross autopsy findings. Included are all deaths clearly indicative of mouse pox, as well as all questionable deaths from unknown causes that could conceivably have been the result of mouse pox. No clearly positive deaths occurred after August, 1953 (*see* text). The 2 mice marked "neg." were tested for ectromelia virus by transfer of liver homogenate to susceptible mice with negative results. During this period the size of the colony varied from approximately 500 to 1,500 mice.

vaccination, only 4 were vaccinated mice, the other 11 being of preweaning age at the time of vaccination of the colony. During the 3 weeks following vaccination, 5 mice died of causes judged by autopsy to be other than mouse pox or the result of vaccination *per se*, 2 mice died with lung congestion as the only gross autopsy finding, and 3 mice were found dead and too much eaten by their cage mates to be autopsied. Only the latter 5 deaths could reasonably be suspected to have possibly resulted from the intranasal instillation of vaccinia virus *per se*. Mortality from mouse pox in the unvaccinated colonies, from which the vaccinated colony had recently been removed, remained high for a somewhat longer period of

TABLE 1.—*Mortality resulting from intranasal inoculation of vaccinia (Levaditi)*

Mouse source or strain	Dilution of inoculum	Mortality (*no. dead/no. inoculated*)
Briody	1:5	1/15
IP Br	1:5	0/2
3 Ca Mg	1:5	3/12
BC	1:5	8/12
BC	1:20	4/8
BC	1:100	0/8
A	1:20	0/5

time and then returned spontaneously to a low level, as with the original outbreak in the summer and fall of 1951.

Because of the disadvantages of immunization by intranasal instillation (need for anesthesia and mortality in certain strains of mice following large doses of vaccinia virus by this route), a study was made of vaccination by dermal scarification, as in smallpox vaccination of humans and as practiced in England (*8*). The base of the tail was scarified and a drop of vaccinia-virus culture applied. This procedure required only a few seconds per mouse. Heavy scarification, which would result in a thin, dark-red, "nonspecific" scab, was found to be more effective than light scarification. If vaccinia virus was applied to the freshly scarified tail of a susceptible mouse, the nonspecific scab was converted to a raised, yellowish, "specific" scab in a period of 1 to 2 weeks (figs. 2 and 3).

Strain A mice in groups of 25 were vaccinated by each of the 2 routes with undiluted vaccinia virus. The mice that received this large dose of vaccinia intranasally looked sick for the next few days, and 9 died with gross pulmonary congestion. This same dose of vaccinia applied to the scarified tails of another group resulted in no mortality and no grossly apparent illness. Both vaccinated groups and 2 groups of unvaccinated controls were challenged with ectromelia virus 26 days later. All but 1 of the 54 controls died of mouse pox, whereas none of the mice vaccinated by either route contracted mouse pox and died (table 2).

On February 11 and 12, 1953, the entire colony was revaccinated by the dermal route, and mice born into the colony thereafter were similarly vaccinated at, or shortly after, the time of weaning. It was found that

TABLE 2.—*Comparison of intranasal* versus *dermal vaccination in strain A mice*

Experimental group	Number of mice	Mortality from vaccinia virus	Nonspecific mortality	Mortality from ectromelia challenge
Controls—no vaccination	29	—	0	29
Controls—tails scarified but not vaccinated	25	—	0	24
Tails scarified and treated with vaccinia virus (Nelson)	25	0	0	0
Intranasal vaccinia virus (Nelson)	25	9	1	0

among mice vaccinated at weaning age fewer developed a vaccinia-specific scab than among mice vaccinated at 6 weeks of age, presumably because of passive transfer of immunity from the mothers. Additional evidence of passive transfer of immunity is suggested by the observation that the the nonvaccinated progeny of vaccinated mice were less susceptible to challenge with ectromelia virus than mice from an "isolation colony" without prior infection with ectromelia virus or vaccination with vaccinia virus (text-fig. 1). Vaccination of all weanling mice at about 6 weeks of age was adopted as a standard procedure. The entire colony was revaccinated again as a routine precaution between April 21 and 28, 1954. After this date, the small percentage of weaners that did not show a vaccinia-specific scab as a result of vaccination at 6 weeks of age were routinely discarded. Commercial calf-lymph vaccine was found to be less effective than egg-cultured vaccine when used without prior egg passage.

Outbreaks of the acute form of mouse pox did not recur subsequent to the routine use of vaccination, but they did recur in the unvaccinated colonies from which this colony had been removed.

Because of the difficulty in diagnosing all deaths from mouse pox by gross autopsy findings only, text-figure 1 includes all mice with autopsy findings clearly indicative of mouse pox as well as all questionable deaths from unknown causes that could conceivably have been the result of mouse pox. After July, 1952, only 3 deaths occurred that were clearly indicative of mouse pox. One of these was in September, 1952, the other 2 in August, 1953.

On February 16, 1953, 48 vaccinated mice, 73 of their unvaccinated progeny, and 67 susceptible mice from the isolation colony were placed on a separate rack in the same room as the rest of the vaccinated colony and challenged with ectromelia virus. As indicated in text-figure 1, 125 of these mice died of the acute form of mouse pox, yet there was no evidence of transmission of the disease to the vaccinated colony. This is in sharp contrast to the progressive spread of the disease throughout the unvaccinated colony during the initial outbreak of 1951 (*6*).

Two similar challenges in July, 1953, however, were followed by and probably responsible for the 2 deaths from mouse pox in the vaccinated colony during August, 1953. Both of these deaths were in the same pen of mice. One was an unvaccinated weanling, the other a vaccinated adult. This pen of mice was eliminated from the colony and no further challenges were performed in the mouse room until the terminal challenge in September, 1954.

During the following year no deaths attributable definitely to mouse pox occurred in the vaccinated colony, and no cutaneous lesions characteristic of the chronic form of mouse pox were seen. Of the 10 questionable deaths that occurred, 2 were tested for ectromelia virus by injection of liver homogenate into susceptible mice, with negative results.

Because mouse pox had disappeared as a clinical entity from the vaccinated colony, an attempt was made to determine whether ectromelia virus might still be present in the environment or as a latent infection.

TABLE 3.—*Ectromelia challenge of September 9, 1954*

Source of mice	Number of mice injected	Specific mortality	
		Number	Percent
Contact test mice	83	83	100
Mice from isolation colony	55	55	100
Mice from main colony not yet vaccinated	21	20	95
Vaccinated mice from main colony	172	9	5

Vaccinia antihemagglutinin titrations could not be used for this purpose, since positive sera result from exposure to either vaccinia or ectromelia virus. Instead, in July, 1954, 82 ectromelia-susceptible mice were introduced into 70 pens of mice of the vaccinated colony. These pens contained 381 mice, both vaccinated adults and unvaccinated weanlings. These mice were allowed to remain in contact for 52 to 65 days. During this period 5 of the susceptible test mice died and were replaced by 1 or more test mice. The cause of death of each of the 5 test mice was carefully determined and, in all cases, proved to be due to causes other than mouse pox. Mouse pox was tested for in all 5 mice by injection of liver homogenate into other susceptible test mice, with negative results. There was no mortality among the new test mice placed in the pens in which the original 5 test mice had died. The contact test mice presumed to be susceptible and other mice of the isolation colony from which they originated were subsequently proved susceptible by challenge with ectromelia virus (table 3). Vaccinated mice from the main colony challenged at the same time were highly resistant, whereas their as yet unvaccinated progeny were highly susceptible.

Summary

An outbreak of mouse pox was effectively controlled by means of cross-immunization with vaccinia virus. Immunization by dermal scarification was equally protective as, and much more practical than, immunization by intranasal instillation of vaccinia virus. Isolation of the colony from outside contacts and vaccination of all mice born into the colony was followed by disappearance of mouse pox as a clinical entity from the colony. Attempts to reveal the latent presence of ectromelia virus gave negative results. Susceptible mice placed in prolonged contact with mice of the vaccinated colony failed to develop mouse pox.

When mouse pox was no longer present in the vaccinated colony and tests for the latent presence of ectromelia virus were negative, mouse pox was still present as a clinical entity in other colonies from which the vaccinated colony had been separated in June, 1952.

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PLATE 65

FIGURE 1.—Mouse that had just died from acute visceral form of mouse pox as a result of injection of ectromelia virus subcutaneously into one of the hind feet 7 days previously. *Note* pale mottling of liver and dark appearance of duodenum (*arrow*) resulting from bloody fluid it contained.

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PLATE 66

FIGURE 2.—Appearance of tail 2 days after vaccination, showing the thin (dark-red) nonspecific scab resulting from scarification.

FIGURE 3.—Appearance of tail 14 days after vaccination, showing conversion of nonspecific scab to a thick (yellowish) vaccinia-specific scab.

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